STUDIES ON THE QUANTITATIVE DIFFERENTIAL ANALYSIS OF MIXTURES OF SEVERAL ESSENTIALLY PURE PENICILLIN TYPES

RICHARD DONOVICK, DANIEL LAPEDES, AND FELIX PANSY

Division of Microbiology, The Squibb Institute for Medical Research, New Brunswick, New Jersey

Received for publication June 24, 1947

Since the work of Schmidt, Ward, and Coghill (1945) on a method for differentiating various types of penicillins by means of two assay organisms, very little further work has been published in this connection. That various organisms respond to different penicillin types differently is well known (Veldee *et al.*, 1945; Welch *et al.*, 1944; Libby and Holmberg, 1945; Eagle, 1946; Eagle and Musselman, 1946; etc.), but few specific attempts have been made to develop a quantitative test for specific penicillin types based on differential response of the organisms studied. A relatively rough *in vivo* differential assay procedure was recently proposed by Buck, Farr, and Schnitzer (1946) in which *Borrelia* infections in mice were used.

With the recognition of the various penicillin types have come improved methods for separating mixtures of penicillins into the pure components (Fischbach *et al.*, 1946; Craig *et al.*, 1946). As these purification methods have been increasingly used, the need for accurate differential biological assay procedures has been felt more and more. The recent publication of Higuchi and Peterson (1947) presents a procedure with which they attempted to fill this need.

The method described by these authors employs three test organisms: *Staphylococcus aureus* 209-P, *Bacillus brevis*, and "organism E." Using a turbidimetric test, they reported that it was possible by their method to estimate with fair accuracy the composition of mixtures consisting of penicillins G, K, and X. They noted that the assay procedure was based on several assumptions, one of which was that "the effects of penicillins in mixtures on assay organisms are additive."

Since systematic studies on this latter question were under way in this laboratory with *Staphylococcus aureus* Heatley, the work was augmented after obtaining cultures of *B. brevis* and "organism E" through the kindness of Dr. Peterson. The present paper includes studies on the effects of known mixtures of several penicillin types on these three species of organisms.

A cursory survey of the literature leads one to believe that activities assigned to each of the various penicillin types actually have been established in some cases with mixtures of several types of penicillin. For example, penicillin F, has been reported to have activities of 1,440 to 1,490 units per mg (Schmidt, Ward, and Coghill, 1945) and 1,550 units per mg (Higuchi and Peterson, 1947). Values of 845 to 935 units per mg (Schmidt *et al.*, 1945), 850 (Coghill and Koch, 1945), 900 (Welch *et al.*, 1944), and 1,000 units per mg (Libby and Holmberg,

1945) have been assigned to penicillin X. The slight shift in the assigned activity of penicillin G from 1,650 units per mg (Welch *et al.*, 1944) to 1,667 units per mg (Veldee *et al.*, 1945) with consequent slight changes in the relative activities of the other penicillin types cannot account for these discrepancies. The various activities reported may be attributable to strains of organisms used for assay, to the assay procedures themselves, or to the varying degrees of purity of the preparations used.

Since the chief purpose of the present work was to study the effect, if any, of one penicillin type on the action of another, efforts were made to use only penicillin preparations the purity of which were as thoroughly established as possible.

PENICILLIN PREPARATIONS USED

Penicillin G[1] (*Cra A-328-36*). This is a crystalline sodium salt prepared from commercial penicillin by chromatography and recrystallized several times. The chemical analysis agreed well with theoretical:

Found: C, 53.81; H, 4.85.
Calc.: C, 53.92; H, 4.81.

Craig countercurrent distribution studies of this preparation revealed that 90 per cent (by weight, 93 per cent of the activity) was contained in the main band, indicating it to be an essentially homogeneous material. The remaining 10 per cent consisted of inactive impurities, possibly inactivation products formed during the distribution experiment.

Bioassays with *Staphylococcus aureus* Heatley against previously well-established standards showed its activity to agree very well with the defined activity of penicillin G, i.e., 1,667 units per mg, suggesting a purity at least as good as any of the materials used in establishing such standards.

Penicillin K[1] (*AV-73*). This is a crystalline ammonium salt obtained by partition chromatography. The chemical analysis agreed well with the theoretical:

Found: C, 53.34, 53.10; H, 8.05, 8.12; N, 11.62; S, 8.92; Moisture, (H_2O) 3.46.
Calc: C, 53.46; H, 8.13; N, 11.69; S, 8.99.

As will be shown later, careful bioassay of this preparation gave an activity of 2,540 units per mg, which is about 10 per cent higher than the figure of 2,300 usually assigned to penicillin K (Coghill and Koch, 1945). The subtilis:staphylococcus ratio of 0.36 was in good agreement with that reported to be characteristic of penicillin K (Coghill and Koch, 1945).

Penicillin X (*NRRL-1717-39A*). The penicillin X used for these studies was supplied us through the kindness of Dr. F. H. Stodola, of the Northern

[1] The authors are indebted to Drs. O. P. Wintersteiner and M. Adler of the Division of Organic Chemistry of the Squibb Institute for Medical Research for the penicillins G and K used in these studies as well as the chemical and physical data describing these preparations.

Regional Research Laboratory, who described it as an analytically pure preparation having the following analysis:

Calc. for $C_{16}H_{17}N_2O_5S$ Na: C, 51.6; H, 4.60.
Found: C, 51.8; H, 4.89.

Their bioassays with *Staphylococcus aureus* (strain not specified) indicated an activity of 920 units per mg.

ASSAY PROCEDURE

Higuchi and Peterson (1947) in their differential assay procedure plotted turbidimetric readings of growth against units of penicillin per ml. When large numbers of assays are involved, a technique similar to that used for streptomycin assay (Donovick *et al.*, 1945) has proved preferable in our hands. It is perhaps true that readings of partial inhibition, as were done by Higuchi and Peterson (1947), may be more accurate when the curves, obtained by plotting turbidimeter readings against units of penicillin per ml, are not steep, which was the case with their strain of *Staphylococcus aureus*. On the other hand, for organisms such as *B. brevis* and "organism E," the curves were very steep, showing a change from little inhibition to almost complete inhibition over a very narrow range of penicillin concentrations. Hence, it appeared to us that little could be gained through the use of a turbidimeter for reading end points. The three test organisms used for the present work were *Staphylococcus aureus* Heatley and, as already indicated, two species used by Higuchi and Peterson (1947), viz., *B. brevis* and "organism E."

Sixteen-hour cultures of the three organisms were diluted as follows: *Staphylococcus aureus* Heatley, 1×10^{-6} in yeast beef broth (Difco); *B. brevis*,[2] 0.25×10^{-5} in "Peterson B"[3] broth; and "organism E", 1×10^{-5} in "Peterson E"[3] broth. These dilutions gave counts of approximately 1,000 organisms per ml.

Two-ml volumes of inoculated broths were dispensed with sterile automatic syringes into sterile tubes measuring 13 by 100 mm. The penicillin solution to be assayed, appropriately diluted, was then added to the 2-ml volumes of inoculated broth by means of acid-cleaned, sterile, 0.2-ml Kahn pipettes in the following amounts: 0.10, 0.088, 0.077, 0.068, 0.059, 0.052, 0.046, 0.040, 0.035,

[2] It was found to be advisable to grow *B. brevis* in a shallow layer of broth to obtain sufficiently heavy growth in 16 hours to allow the indicated dilution for the tests.

[3] These media were used by Dr. Peterson in some of his early work and were recommended to us by him (personal communication). They had the following compositions:

	Peterson "B" broth g/liter	*Peterson "E" broth g/liter*
Peptone	6.0	6.0
Yeast extract (Difco)	3.0	3.0
Glucose	1.0	2.0
K_2HPO_4	3.2	0.5
KH_2PO_4	2.0	5.0
pH	6.8	6.0

and 0.030 ml. The racks containing the tubes of inoculated broth were kept at 5 C prior to the addition of penicillin. Three racks at a time (i.e., one rack of each of the three test organisms) were removed from the icebox, the penicillin was added, and the racks were returned at once to the cold room (5 C). When penicillin had been added to all the racks for a given day, they were all placed in the appropriate incubators[4] at one time and incubated for 15½ to 16½ hours.

The tests, after the tubes were vigorously shaken, were read under a fluorescent day lamp. Absence of growth was recorded as (−), an intermediate degree of growth as (±), and almost complete or complete growth as (+). The end point was considered to be the last (−) in a (−) (+) series; and the midpoint between (−) and (±) in a (−) (±) (+) series. Since in the present investigations the concentrations (by weight) of penicillin in the solutions tested were known, the minimal inhibiting concentrations (M.I.C.) were readily calculated from the volume of penicillin solution added to the end point tube.

Early in the present studies aqueous solutions of each type of penicillin were prepared from carefully weighed samples. The desired mixtures were made by mixing appropriate proportions of the various solutions. All samples were then dispensed in acid-cleaned, sterile ampoules, in ca. 1-ml amounts, and the ampoules sealed and frozen in a CO_2-alcohol bath. The ampoules were then stored in a CO_2 box until used. When assays were to be made, enough ampoules for that days' work were thawed, and the contents were diluted with distilled water and assayed.

It will be noted that Higuchi and Peterson (1947) expressed penicillin concentrations in terms of the standard unit, "in order to compare the results obtained . . . with previous results." As a consequence, the algebraic expressions which they derived for calculating compositions of penicillin mixtures yielded results in units per cent. The present authors feel that where three test organisms and three or more types of penicillin are involved the use of units leads to confusion and obscures various relationships. This will be discussed more fully below, but suffice it to say for the moment that all M.I.C. data were gathered and are here reported in terms of actual weights of penicillin per unit volume, and the equations given below yield results in percentage by weight. It is obvious that the composition of a mixture containing, e.g., 50 per cent G and 50 per cent K by weight is quite different from one the activity of which consists of 50 units of G and 50 units of K.

Tests on known mixtures of two or more types of penicillin were always accompanied by controls consisting of tests on solutions containing separately the individual components involved in the mixtures. Hence, large numbers of assays of the solutions containing only single types of penicillin were conducted. In table 1 are shown the results of the tests on these control solutions.

Comparison of the M.I.C. values shown in table 1 with those given by Higuchi and Peterson (1947) reveals surprising differences in findings. The cause of these differences is uncertain, but several explanations suggest themselves.

[4] *Staphylococcus aureus* and *B. brevis* were incubated at 37 C; "organism E" at 45 C.

TABLE 1

Minimal inhibiting concentrations of penicillin in terms of weight

PENICILLIN	EXPERIMENT NO.	M.I.C.		
		S. aureus (Heatley)	*B. brevis*	"Organism E"
		μg/ml	μg/ml	μg/ml
G (CrA-328-36)	1	0.00790 (27)*	0.0125 (25)	0.0311 (23)
G (CrA-328-36)	2	0.00770 (126)	0.0150 (120)	0.0308 (120)
G (CrA-328-36)	3	0.00720 (65)	0.0145 (60)	0.0329 (55)
Average G.....		0.00755 ± 0.735%†	0.0146 ± 0.957%	0.0317 ± 0.897%
K (AV-73)	1	0.00463 (8)	0.0458 (8)	0.0686 (8)
K (AV-73)	2	0.00515 (31)	0.0475 (28)	0.0690 (29)
K (AV-73)	3	0.00480 (24)	0.0458 (26)	0.0641 (25)
Average K.....		0.00495 ± 1.29%	0.0465 ± 1.17%	0.0670 ± 2.14%
X (NRRL-1717-39A)	1	0.0136 (51)	0.0535 (49)	0.0283 (43)
X (NRRL-1717-39A)	2	0.0133 (26)	0.0535 (22)	0.0277 (24)
X (NRRL-1717-39A)	3	0.0143 (24)	0.0585 (24)	0.0289 (24)
X (NRRL-1717-39A)	4	0.0154 (30)	0.0550 (29)	0.0342 (31)
Average X.....		0.0140 ± 1.04%	0.0545 ± 1.10%	0.0295 ± 1.49%

* Figure in parenthesis represents number of assays conducted on the specific sample.

† The standard errors shown were calculated on the results of the total number of assays carried out with a specific preparation. The authors are indebted to Mr. Ross Blue of E. R. Squibb & Sons for this statistical analysis.

It is possible that slight differences in media, or perhaps in variations occurring in the cultures, between the time Higuchi and Peterson conducted their tests and the time we received these cultures may have accounted in part for these differences. Probably even more important was that in the present work complete inhibition was taken as the end point, whereas some point of partial inhibition (but which is not clearly indicated) was used as the end point by Higuchi and Peterson (1947).

The latter authors reported M.I.C. values, for their strain of *Staphylococcus aureus* (209-P), which are close to twice as great as those found for *Staphylococcus aureus* Heatley in the present work. Though the strains of *B. brevis* and "organism E" used for these studies were the same as those used by the foregoing authors, the minimal inhibiting concentrations reported by them for the various penicillin types studied are quite different from those reported here. It is interesting, therefore, to note how the results compare when the present data are converted into units.

Assigning to penicillin G its defined activity of 1,667 units per mg, the K penicillin used in the present work would have an activity of $1{,}667 \times \frac{7.55}{4.95} =$ 2,540 units per mg, and the X used would have $1{,}667 \times \frac{7.55}{14.0} = 898$ units per

TABLE 2

Minimal inhibiting concentrations of penicillin in terms of units

TEST ORGANISM	PENICILLIN TYPE	ACTIVITY OF PENICILLIN UNITS PER MG	M.I.C.		RATIO OF M.I.C.'S IN TERMS OF UNITS	
			μg per liter	Units per liter	G/K	G/X
S. aureus (Heatley)	G	1,667	7.55	12.6		
S. aureus (Heatley)	K	2,540	4.95	12.6	1	
S. aureus (Heatley)	X	898	14.0	12.6		1
B. brevis	G	1,667	14.6	24.3		
B. brevis	K	2,540	46.5	118.1	0.206	
B. brevis	X	898	54.5	48.9		0.497
"Organism E"	G	1,667	31.7	52.8		
"Organism E"	K	2,540	67.0	170.1	0.310	
"Organism E"	X	898	29.5	26.5		1.97

mg. On the basis of these potencies, conversion of the M.I.C. values shown (in terms of weight) in table 1 to M.I.C. in terms of units give the results shown in table 2.

Thus, despite the differences between the absolute (weight) M.I.C. values reported here and by Higuchi and Peterson (1947), equations very similar to those of the latter authors, based on relative (unitage) M.I.C. ratios, may be set up.

STUDIES WITH KNOWN MIXTURES CONTAINING TWO TYPES OF PENICILLIN

To determine whether the effects of the various types of penicillins were truly additive, mixtures containing two types were first studied. The findings with such two component mixtures are shown in tables 3, 4, and 5. Studies were then undertaken with three component mixtures and these results are listed in table 6. The values listed under the columns headed "theor." were calculated by means of equations (1), (2), and (3) below after substituting in the known values of a, b, and c. These equations hold true only in so far as the effects of the penicillins are additive, in which case there would be a direct proportionality between the composition of a given mixture and the M.I.C. values of this mixture for the three test organisms.

The following equations relate the concentration (in terms of weight per volume) of mixed penicillins at the end point to the composition of the mixture:

In a mixture of penicillins, let—

a = per cent, by weight, of penicillin G

b = per cent, by weight, of penicillin K

c = per cent, by weight, of penicillin X

MsG = M.I.C. of pure penicillin G for *Staphylococcus aureus*

MsK = M.I.C. of pure penicillin K for *Staphylococcus aureus*

MsX = M.I.C. of pure penicillin X for *Staphylococcus aureus*

Similarly let—
MbG = M.I.C. of pure penicillin G for *Bacillus brevis*
MeG = M.I.C. of pure penicillin G for "organism E," etc.
Ms = M.I.C. of mixture of penicillin for *Staphylococcus aureus*
Mb = M.I.C. of mixture of penicillin for *Bacillus brevis*, etc.

TABLE 3

Minimal inhibiting concentrations of known mixtures of penicillins G and K

COMPOSITION* OF MIXTURE		S. AUREUS			B. BREVIS			"ORGANISM E"		
		Ms μg per liter			Mb μg per liter			Me μg per liter		
Per cent G	Per cent K	Theor.	Found	Found / Theor.	Theor.	Found	Found / Theor.	Theor.	Found	Found / Theor.
100	0	7.55†	7.55	1.00	14.6†	14.6	1.00	31.7†	31.7	1.00
90	10	7.16	7.50	1.05	15.7	15.5	0.99	33.4	35.5	1.06
80	20	6.83	7.00	1.02	16.9	17.5	1.03	35.4	37.5	1.06
65	35	6.38	7.18	*1.12*	19.2	20.0	1.04	38.9	44.3	*1.14*
50	50	5.98	7.01	*1.17*	22.2	22.0	0.99	43.1	49.6	*1.15*
35	65	5.63	6.75	*1.20*	26.4	28.5	1.08	48.3	54.6	*1.13*
20	80	5.31	6.34	*1.19*	32.4	33.5	1.03	54.7	55.0	1.00
10	90	5.12	5.50	1.07	38.2	39.0	1.02	60.4	65.0	1.08
0	100	4.95†	4.95	1.00	46.5†	46.5	1.00	67.0†	67.0	1.00

Figures in italics indicate that the data from which the ratios were derived were analyzed statistically and that the deviations from 1.00 were significant.

* Composition in terms of grams of given penicillin per 100 grams of total penicillin.

† Theoretical figure for solution containing only one component is assigned by definition and is equal to the experimentally determined end point.

TABLE 4

Minimal inhibiting concentrations of known mixtures of penicillins G and X

COMPOSITION* OF MIXTURE		S. AUREUS			B. BREVIS			"ORGANISM E"		
		Ms μg per liter			Mb μg per liter			Me μg per liter		
Per cent G	Per cent X	Theor.	Found	Found / Theor.	Theor.	Found	Found / Theor.	Theor.	Found	Found / Theor.
100	0	7.55†	7.55	1.00	14.6†	14.6	1.00	31.7†	31.7	1.00
90	10	7.92	8.82	*1.11*	15.8	17.0	1.08	31.4	35.4	*1.18*
80	20	8.32	8.87	*1.06*	17.1	17.5	1.02	31.2	34.0	*1.09*
65	35	9.00	9.00	1.00	19.6	20.5	1.02	30.8	33.5	1.09
50	50	9.84	10.5	1.07	23.0	22.5	0.98	30.5	32.5	1.06
35	65	10.8	11.5	*1.07*	28.8	27.0	0.94	30.2	31.6	1.04
20	80	12.0	13.3	*1.11*	35.2	36.0	1.02	29.9	31.9	*1.06*
10	90	12.9	13.9	*1.08*	42.7	43.0	1.01	29.7	32.5	*1.09*
0	100	14.0†	14.0	1.00	54.5†	54.5	1.00	29.5†	29.5	1.00

Figures in italics indicate that the data from which the ratios were derived were analyzed statistically and that the deviations from 1.00 were significant.

* Composition in terms of grams of given penicillin per 100 grams of total penicillin.

† Theoretical figure for solution containing only one component is assigned by definition and is equal to the experimentally determined end point.

Then,[5]

$$(1)\qquad Ms = \frac{100}{\frac{a}{MsG} + \frac{b}{MsK} + \frac{c}{MsX}} = \frac{100}{\frac{a}{7.55} + \frac{b}{4.95} + \frac{c}{14.0}}$$

$$(2)\qquad Mb = \frac{100}{\frac{a}{MbG} + \frac{b}{MbK} + \frac{c}{MbX}} = \frac{100}{\frac{a}{14.6} + \frac{b}{46.5} + \frac{c}{54.5}}$$

$$(3)\qquad Me = \frac{100}{\frac{a}{MeG} + \frac{b}{MeK} + \frac{c}{MeX}} = \frac{100}{\frac{a}{31.7} + \frac{b}{67.0} + \frac{c}{29.5}}$$

$$(4)\qquad a + b + c = 100.$$

TABLE 5

Minimal inhibiting concentrations of known mixtures of penicillin K and X

COMPOSITION* OF MIXTURE		S. AUREUS			B. BREVIS			"ORGANISM E"		
		Ms μg per liter			Mb μg per liter			Me μg per liter		
Per cent K	Per cent X	Theor.	Found	Found / Theor.	Theor.	Found	Found / Theor.	Theor.	Found	Found / Theor.
100	0	4.95†	4.95	1.00	46.5†	46.5	1.00	67.0†	67.0	1.00
90	10	5.28	5.50	1.04	47.2	47.3	1.00	59.5	62.5	1.05
80	20	5.69	5.44	0.96	47.8	54.1	*1.13*	53.5	49.0	0.92
65	35	6.41	6.50	1.01	49.0	56.6	*1.15*	46.5	45.5	0.98
50	50	7.38	8.15	*1.10*	50.2	56.7	*1.13*	41.0	47.8	*1.16*
35	65	8.54	9.00	1.05	51.4	55.0	1.07	36.8	38.6	1.02
20	80	10.3	11.0	1.07	52.6	54.3	1.03	33.2	32.2	0.97
10	90	11.9	12.5	1.05	53.6	56.5	1.05	31.2	33.0	1.06
0	100	14.0†	14.0	1.00	54.5†	54.5	1.00	29.5†	29.5	1.00

Figures in italics indicate that the data from which the ratios were derived were analyzed statistically and that the deviations from 1.00 were significant.

* Compositions in terms of grams of given penicillin per 100 grams of total penicillin.

† Theoretical figure for solution containing only one component is assigned by definition and is equal to the experimentally determined end point.

The ratio between the "theoretical" M.I.C. and the experimentally determined M.I.C. is a measure of the extent to which the effects of the various penicillin types are additive since, as already indicated, the equations above are based on the assumption that they are additive. Hence, the "theoretical" value and the experimentally determined value for a given mixture should be equal, within experimental error, when the effects are additive.

It was found that with certain two component mixtures, the experimental M.I.C. values were as high as 20 per cent greater than expected. This would

[5] For convenience in handling the figures, the M.I.C. values are here given in terms of μg per liter.

indicate that a certain amount of interference with the action of one penicillin is caused by the presence of a second penicillin.

It may well be asked whether these deviations from the theoretical M.I.C.'s are significant. Handicaps in biological assay procedures at best are the relatively large standard deviations which occur unless a large number of assays are done. Consequently, in order to determine whether these observed deviations were statistically significant, a great many of the data involved were subjected to statistical analysis.[6] The findings of this analysis are shown in figure 1 and indicate that the deviations are significant.[7] In two component mixtures consisting of penicillins G and K, significantly larger amounts of penicillin were

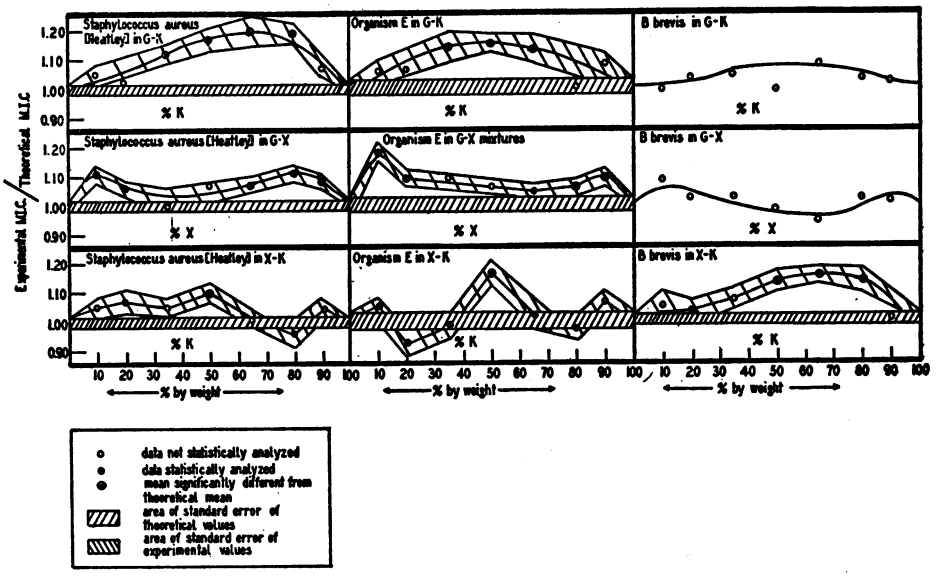

FIG. 1. DEVIATION OF EXPERIMENTAL M.I.C. VALUES FROM THEORETICAL VALUES IN TWO-COMPONENT MIXTURES OF PENICILLIN

required to inhibit *Staphylococcus aureus* Heatley and "organism E" than would be expected on the basis of the M.I.C. values of the individual penicillins. This is especially evident in mixtures containing between 35 and 65 per cent K. Although the deviation from theory for such mixtures in the case of *B. brevis*

[6] The authors are indebted to Mr. Ross Blue of the Division of Product Control of the Chemical and Biological Laboratories, E. R. Squibb & Sons, for carrying out this task.

[7] In accordance with common practice, the difference between a given mean experimental M.I.C. value and the corresponding theoretical value was considered significant when this difference was at least two times as great as the standard error of the difference. In the cases considered as significant in figure 1, the differences between experimental and theoretical M.I.C. values were from 2.1 to 5.1 times as great as the standard errors of the differences. (For a complete discussion on such statistical procedures, see such standard texts as F. C. Mills, *Statistical Methods*, Henry Holt and Co., New York, 1939.)

is apparently not statistically significant, yet the tendency appears to be in the same direction.

When the mixture consisted of penicillins G and X, deviations reached maxima in two regions, one in the vicinity of 10 per cent X and another at 80 to 90 per cent X for both *Staphylococcus aureus* Heatley and "organism E." The picture appeared to be similar here in the case of *B. brevis*, but again the deviations from theory were not statistically significant.

The data on staphylococcus and "organism E" in K-X mixtures were very inconclusive except in the vicinity of 50-50 mixtures in which the amount of penicillin required to inhibit was again significantly greater than expected. In this case the data on *B. brevis* were quite clear-cut. Significantly more penicillin was required to inhibit this organism than would be expected in mixtures covering the range of 50 to 80 per cent X.

It is of interest to note that in none of the cases studied was the experimental M.I.C. significantly less than the theoretical figure. It would appear, therefore, that in two component mixtures of penicillins, one penicillin may interfere with the action of the other, thereby requiring a greater total amount of penicillin to cause inhibition than might be expected. Since very little is understood of the mode of action of the penicillins, it is not possible at present to explain this apparent interference. It is not even clear whether these compounds act within the bacterial cell or upon the cell surfaces, nor, in fact, whether all the penicillins inhibit growth in identically the same fashion.

If, as a working hypothesis, one were to assume that the penicillins act within the cell rather than upon the surface, then one might tentatively propose that the apparent interference may actually be caused by differential adsorption of the various penicillins at the cell surface as well as differential diffusion into the cell. This would result in the composition of the penicillin mixture inside the cell being different from that outside. For example, it can be seen in table 3 that for *Staphylococcus aureus* Heatley the experimental M.I.C. of a mixture containing 20 per cent G and 80 per cent K is equal to the theoretical M.I.C. of a 65 per cent G and 35 per cent K mixture. On the other hand, for "organism E" the experimental M.I.C. of a mixture containing 35 per cent G and 65 per cent K is equal to the theoretical M.I.C. of a 20 per cent G and 80 per cent K mixture. If differential adsorption is in fact the reason for the observed interference, then one might expect penicillin K to be adsorbed more readily than penicillin G by *Staphylococcus aureus* Heatley and the reverse to be true for "organism E." Studies on adsorption of penicillin by bacteria which are under way in this laboratory (Rake *et al.*, to be published) may perhaps lend weight for or against such a hypothesis.

Of course, the interference may be due to competition at a site of action of penicillin within the bacterial cell, but little can be said about this in the present state of knowledge of the mode of action of the penicillins.

STUDIES WITH MIXTURES CONTAINING THREE TYPES OF PENICILLIN

The question of deviation of behavior from the expected becomes increasingly difficult to answer with the increase in the number of penicillins involved. It

was assumed, for purposes of calculation, that the effects of the penicillins were additive, and equations were derived expressing the relationship between the M.I.C. of a mixture and its composition by weight—equations (1), (2), (3), and (4). Using these equations the "theoretical" values of Ms, Mb, and Me for 10 three-component mixtures were calculated, and at the same time these values were determined experimentally for these mixtures. The comparison of these two sets of data is shown in table 6.

It will be noted that with *Staphylococcus aureus* and *B. brevis* the ratios between "theoretical" and experimental M.I.C. values were usually very close to 1.0, whereas with "organism E" in 5 out of 10 mixtures the ratios indicated

TABLE 6

Minimal inhibiting concentrations of known mixtures of penicillins G, K, and X

COMPOSITION* OF MIXTURE			S. AUREUS			B. BREVIS			"ORGANISM E"		
			Ms μg per liter			Mb μg per liter			Me μg per liter		
Per cent G	Per cent K	Per cent X	Theor.	Found	Found / Theor.	Theor.	Found	Found / Theor.	Theor.	Found	Found / Theor.
100	0	0	7.55†	7.55	1.00	14.6†	14.6	1.00	31.7†	31.7	1.00
0	100	0	4.95	4.95	1.00	46.5	46.5	1.00	67.0	67.0	1.00
0	0	100	14.0	14.0	1.00	54.5	54.5	1.00	29.5	29.5	1.00
80	10	10	7.50	7.50	1.00	17.0	16.5	0.97	33.2	38.0	1.14
60	20	20	7.45	7.50	1.01	20.4	19.5	0.96	34.9	36.5	1.05
40	40	20	6.75	6.50	0.96	25.2	25.0	0.99	39.5	39.5	1.00
40	20	40	8.19	8.50	1.04	25.6	25.5	1.00	34.4	38.0	1.10
33.3	33.3	33.3	7.39	7.50	1.01	27.7	25.5	0.92	37.3	39.0	1.05
20	60	20	6.17	6.50	1.05	33.0	35.5	1.07	45.5	48.0	1.05
20	40	40	7.36	8.00	1.09	33.8	37.0	1.09	38.8	44.0	1.13
20	20	60	9.11	9.40	1.03	34.5	33.5	0.97	33.7	38.0	1.13
10	10	80	11.0	11.5	1.04	42.2	43.4	1.03	31.5	37.0	1.17
10	80	10	5.49	4.95	0.90	38.6	37.5	0.97	54.0	55.0	1.02

* Composition in terms of grams of given penicillin per 100 grams total penicillin.

† Theoretical figure for solution containing only one component is assigned by definition and is equal to the experimentally determined end point.

that from 10 to 17 per cent more penicillin than expected was required. Although the experimental error in this work was probably no greater than that with the two component mixtures (since the two sets of data were gathered under identical conditions), the error in the "theoretical" figures would be statistically higher, having been derived algebraically from data on each of the three types of penicillin used, and the total error would contain errors from figures on each type of penicillin. Consequently, no attempts were made to establish the degree of significance of these deviations. Instead, attention was turned to the question of how well the compositions of the various mixtures could be calculated from the experimental data.

Going back to equations (1), (2), (3), and (4) it can be seen that, by solution with simultaneous equations, the concentration (per cent by weight of total

penicillin) of each component in any mixture can be expressed in three ways, i.e., in terms of Ms and Mb, Ms and Me, or Mb and Me. Solutions in terms of two of these three combinations are shown in the following equations:

$$a = \frac{2{,}054}{Mb} - \frac{49.6}{Ms} - 34.16 \tag{5}$$

$$b = \frac{789}{Ms} - \frac{961}{Mb} - 38.69 \tag{6}$$

$$c = 172.8 - \frac{1{,}093}{Mb} - \frac{739}{Ms} \tag{7}$$

or

$$a' = \frac{15{,}320}{Me} + \frac{2{,}226}{Ms} - 678.5 \tag{8}$$

$$b' = 262.7 - \frac{273.7}{Ms} - \frac{7{,}169}{Me} \tag{9}$$

$$c' = 515.7 - \frac{8{,}155}{Me} - \frac{1{,}951}{Ms} \tag{10}$$

Thus theoretically it should be possible to determine the composition of a three-component mixture through the use of two test organisms rather than three.

When the "theoretical" values for Ms, Mb, or Me, as the case may be, are substituted into the foregoing equations, the correct values for a, b, and c (or a', b', and c') are of course obtained. In table 7 are shown the results when the experimentally determined M.I.C. values are used in these equations. Considering that the experimental M.I.C. values, even with "organism E," exceeded the "theoretical" value by as much as 15 per cent in only one case, it was at first surprising to find that the use of equations (8), (9), and (10)—which involve the use of Ms and Me—yielded such meaningless results for a', b', and c' (i.e., per cent G, per cent K, per cent X). However, examination of these data with the aid of triangular co-ordinate graph paper (Keuffel and Esser Co., no. 359-32) supplied the explanation for such results.

Equations (1), (2), and (3) may be expressed:

$$a/7.55 + b/4.95 + c/14.0 = 100/Ms \tag{11}$$

$$a/14.6 + b/46.5 + c/54.5 = 100/Mb \tag{12}$$

$$a/31.7 + b/67.0 + c/29.5 = 100/Me \tag{13}$$

Since $a + b + c = 100$, each of these equations may further be expressed in terms of two unknowns instead of three, again through solution with simultaneous equations:

$$0.0610\,a + 0.1305\,b = 100/Ms - 7.15 \tag{14}$$

$$0.0470\,b + 0.05015\,c = 6.85 - 100/Mb \tag{15}$$

$$0.0166\,a + 0.0190\,c = 100/Me - 1.49 \tag{16}$$

Each of these equations yields an infinite series of parallel straight lines when plotted on triangular co-ordinate paper, the location and direction of each line depending upon the equation and M.I.C. value (i.e., Ms, Mb, or Me) involved. With a given three-component mixture of penicillins, having "theoretical" M.I.C. values of, e.g., Ms_1, Mb_1, and Me_1, the composition of the mixture will be given by the location of the point of intersection of the three lines formed by the plotting of equations (14), (15), and (16) into which the given M.I.C. values have been substituted.

TABLE 7

Compositions of mixtures containing three types of penicillin as calculated from minimal inhibiting concentrations

KNOWN COMPOSITION* OF MIXTURE			COMPOSITION CALCULATED ON BASIS OF EXPERIMENTAL M.I.C.					
Per cent G	Per cent K	Per cent X	a†	b†	c†	a'‡	b'‡	c'‡
100	0	0	100.0	−0.3	0.1	99.5	0.4	0.1
0	100	0	−0.1	100.1	0	−0.3	100.4	−0.1
0	0	100	−0.1	0.2	100.0	−0.3	0.2	100.0
80	10	10	83.6	8.3	8.1	21.3	38.6	40.3
60	20	20	64.5	17.2	18.2	38.0	29.8	32.3
40	40	20	40.3	44.2	15.9	51.7	39.1	9.3
40	20	40	40.5	16.5	43.1	−13.0	41.9	70.9
33.3	33.3	33.3	39.6	28.9	31.5	11.3	42.4	46.6
20	60	20	15.8	55.7	28.4	−17.0	71.3	45.8
20	40	40	15.1	34.0	50.9	−52.1	65.6	86.8
20	20	60	21.9	16.5	61.9	−38.6	45.0	93.4
10	10	80	8.8	7.8	83.3	−71.0	45.3	125.3
10	80	10	10.6	95.1	−5.3	49.0	78.0	−26.6

* Composition in terms of grams of given penicillin per 100 grams total penicillin.

† Calculated by use of equations (5), (6), and (7).

‡ Calculated by use of equations (8), (9), and (10).

An example of the use of this graphic procedure is shown in figure 2. For this example a mixture consisting of 40 per cent G, 20 per cent K, and 40 per cent X was used. As was shown in table 6, the "theoretical" M.I.C. values of such a mixture would be Ms = 8.19, Mb = 25.6, and Me = 34.4 μg per liter, respectively. These values were substituted in equations (14), (15), and (16), and the lines drawn after locating for each line two points through which it passed. (In most cases it is simplest to calculate the value of b, when a = 0, and the value for a, when b = 0, etc. This locates two points on opposite borders of the graph through which the given line passes.)

In figure 2 the solid lines are those obtained by plotting the equations into which have been substituted the "theoretical" M.I.C. values given above. It can be seen that the three lines intersect at the point when a = 40, b = 20, and c = 40. Of course, plottings of the three equations will give lines which will intersect at precisely the correct point only so long as the three M.I.C. values are precisely correct. Since the latter are experimentally determined,

such precision cannot be expected. Hence, it is of interest to know how changes in the M.I.C. values will affect the location of the point or points of intersection. To demonstrate this effect each of the three M.I.C. values used in plotting the lines of figure 2 were arbitrarily increased by 10 per cent and new lines plotted corresponding to these new M.I.C. values. This gave the dotted lines shown in figure 2.

It is at once evident that a 10 per cent error in both Ms and Mb does not shift the point of intersection of these lines nearly so much as it shifts the point of intersection of the Ms and Me lines. In fact, an error of 10 per cent in the

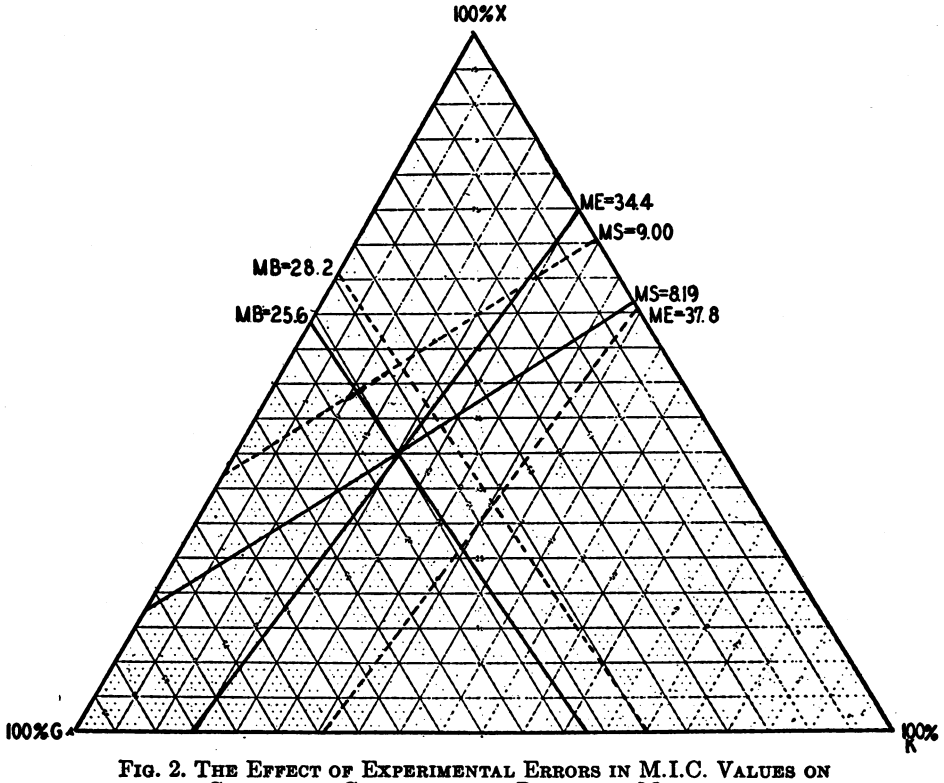

FIG. 2. THE EFFECT OF EXPERIMENTAL ERRORS IN M.I.C. VALUES ON CALCULATED COMPOSITION OF PENICILLIN MIXTURES

Me value causes its line to intersect the Ms line somewhere off the graph (at an imaginary point, since in the case demonstrated this would mean a negative value for a, or less than 0 per cent penicillin G). Examination of this graph indicates that *Staphylococcus aureus* and "organism E" make a poor pair for quantitative differential analysis of penicillin mixtures. *Bacillus brevis* and "organism E" are a somewhat better pair, but the best pair here tested is *Staphylococcus aureus* and *B. brevis*. The graph also indicates why the use of the experimentally determined Ms and Me values (table 6) when used in equations (8), (9), and (10) gave such meaningless values for a′, b′, and c′ (table 7). It

can also be seen why the use of the experimentally determined Ms and Mb values gave fairly good figures for a, b, and c (table 7) when equations (5), (6), and (7) were employed. Thus the use of triangular co-ordinate graph paper in this manner could be of great aid in the search for organisms best suited for quantitative differential analysis of penicillin mixtures.

The 10 three-component mixtures which were studied were analyzed graphically in this manner, calculating the composition from the experimentally determined Ms and Mb values. In figure 3 the compositions calculated in this

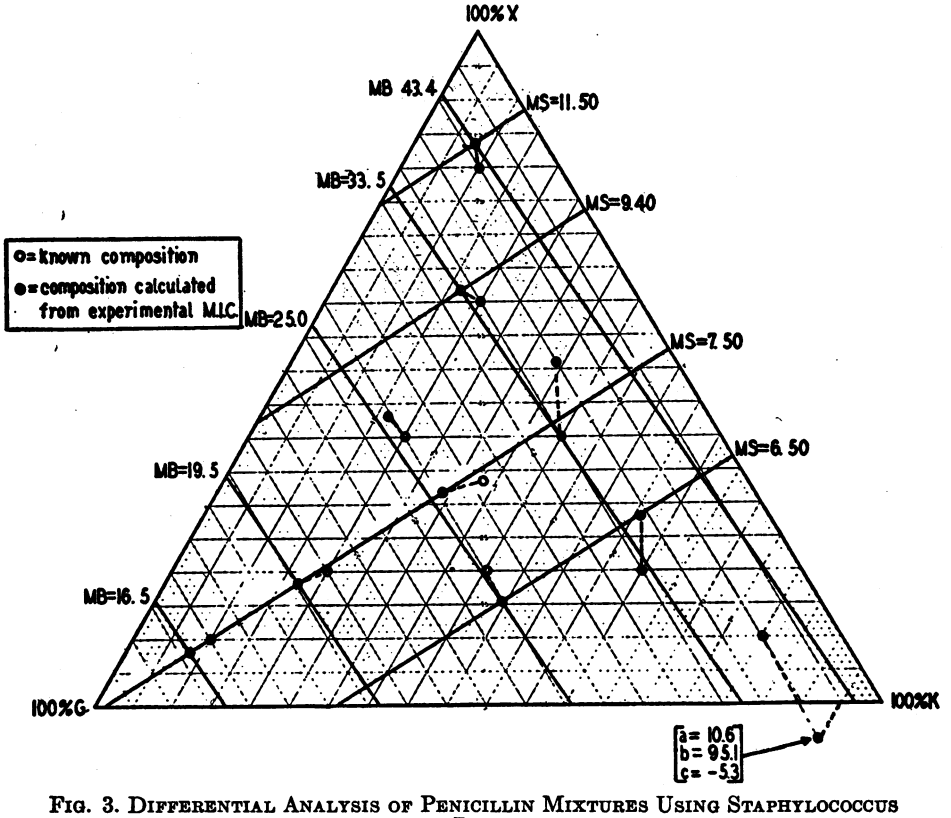

FIG. 3. DIFFERENTIAL ANALYSIS OF PENICILLIN MIXTURES USING STAPHYLOCOCCUS AUREUS AND BACILLUS BREVIS

manner are shown as solid points, and the known composition of the mixtures are shown as open circles. To simplify the appearance of this graph for purposes of photography some of the Ms and Mb lines have been omitted, but their points of intersection (solid dots) are shown.

It will be noted that only when the content of penicillin G fell below ca. 20 per cent of the total penicillin present in a mixture were there marked differences between the estimated and known compositions. Even these differences may have been within experimental error, except for the case of the mixture consisting of 10 per cent G, 80 per cent K, and 10 per cent X. In this case the Ms value was equal to that for pure K. This resulted in the point, representing

the composition of the mixture, falling outside the limits of the graph. For all mixtures containing more than 20 per cent G, the results were probably all within the range of experimental error.

From the data thus far obtained it has not yet been possible to demonstrate a clear-cut interference of one penicillin with another in three-component mixtures. Hence it is not yet clear what effect the addition of a third penicillin component has on the behavior of an existing two-component system. It appears safe to say, however, that differential penicillin bioassays as they now exist yield only rough approximations of the composition of mixtures even when they consist only of penicillins G, K, and X. The presence of more than three penicillins in a mixture results in even less accuracy in its differential assay results. Greatest value may be derived from such procedures in studying the final purification of a given penicillin species when it is already almost pure, or when considerable chemical data have been gathered on a preparation to show that it consists of no more than three types of penicillin.

It perhaps should be pointed out, in passing, that there are certain real advantages in determining the M.I.C. of relatively pure preparations in terms of weight rather than in units. For example, Hobby *et al.* (1946), in studying several types of penicillin, indicated that the bacterial spectrum of these preparations yielded little information and that only with certain strains of *Eberthella typhosa* was it possible to demonstrate a difference in any of the forms of penicillin. This is true only so long as the activities of the various penicillins are expressed in units, and is so of necessity for *Staphylococcus aureus* by the definition of the unit itself. When the standard unit is used, all penicillin activities are expressed in terms of a measure of the sensitivity of *Staphylococcus aureus* to penicillin G. It can be seen in table 2 that, despite the distinct differences in sensitivity of *Staphylococcus aureus* Heatley to penicillins G, K, and X in terms of weight, these differences are hidden when the M.I.C.'s are given in terms of units. If the M.I.C.'s are given in weights, essentially pure penicillin preparations are readily identified by ratios of M.I.C.'s. Thus the characteristic ratio for penicillin G is:

Staphylococcus: brevis: "organism E": = 7.55:14.6:31.7 = *1:1.93:4.2*

For penicillin K it is:

$$4.95:46.5:67.0 = 1:9.4:13.5$$

and for penicillin X:

$$14.0:54.5:29.5 = 1:3.9:2.1$$

Or the same data could be presented in another fashion: the K:G M.I.C. ratio for *Staphylococcus aureus* Heatley is 0.655; that of X:G is 1.86. Similarly for *B. brevis*, K:G is 3.18; X:G is 3.73. And for "organism E," K:G is 2.15 and X:G is 0.925. Some studies were also conducted with several penicillin F preparations, but, since there was some doubt as to the purity of the materials available, these data have not been reported here. It appears, however, that

the staphylococcus: brevis: "organism E" ratio of penicillin F is in the vicinity of 8.4:32:58 = *1:3.8:6.9*.

In the final purification of preparations of the various penicillin types these triple ratios, as has become the custom to call them, have been of considerable aid as guidelines.

SUMMARY

Using *Staphylococcus aureus* Heatley, *Bacillus brevis*, and Peterson's "organism E" as test organisms, it has been shown that in mixtures of two types of penicillin, more penicillin is required to cause inhibition of growth than would be expected from data on the actions of the individual penicillin types. Until the mode (or modes) of action of the penicillins are better understood, this interference on the part of one type of penicillin with the action of another cannot be explained. However, it is tentatively proposed that this phenomenon may be caused by differential adsorption of the various penicillins at the cell surface as well as differential diffusion into the cell.

Equations are given which show the algebraic relationship between the composition of a given penicillin mixture and the weight of total mixed penicillin required to inhibit growth. Through the use of these equations, as well as through the use of a graphic procedure employing triangular co-ordinate paper, it has been shown that only two test organisms are needed for the analysis of mixtures containing three types of penicillin and that *Staphylococcus aureus* Heatley and "organism E" make a poor pair of organisms for such quantitative differential analyses. A better pair of test organisms is that of *Staphylococcus aureus* Heatley and *B. brevis*. However, evidence is also presented to show that even with this pair of organisms relatively slight variations in the experimentally determined minimal inhibiting concentrations cause significant variations in the calculated composition of such mixtures. Hence, such procedures at best give only rough approximations of the composition of penicillin mixtures and are most valuable in the final purification steps of single penicillin types.

The graphic procedure described may prove to be of assistance in finding the best test organisms for such differential analyses.

It has been pointed out that when essentially pure penicillins are involved there are advantages in calculating minimal inhibiting concentrations in terms of weight instead of in units.

REFERENCES

Buck, M., Farr, A. C., and Schnitzer, R. J. 1946 The differentiation of penicillins G and K by an assay method *in vivo*. Science, **104**, 370–371.

Coghill, R. D., and Koch, R. S. 1945 Penicillin. A wartime accomplishment. Chem. Eng. News, **23**, 2310–2316.

Craig, L. C., Hogeboom, G. H., du Vigneaud, V., and Carpenter, F. H. 1946 Separation and characterization of the penicillins by the method of counter-current distribution. Unpublished report distributed by the Research Grants Office, National Institute of Health.

Donovick, R., Hamre, D., Kavanagh, F., and Rake, G. 1945 A broth dilution method of assaying streptothricin and streptomycin. J. Bact., **50**, 623-628.

EAGLE, H. 1946 The relative activity of penicillins F, G, K, and X against spirochetes and streptococci *in vitro*. J. Bact., **52,** 81–88.

EAGLE, H., AND MUSSELMAN, A. 1946 The low therapeutic activity of penicillin K relative to that of penicillins F, G, and X and its pharmacological basis. Science, **103,** 618–620.

FISCHBACH, H., MUNDELL, M., AND EBLE, T. E. 1946 Determination of penicillin K by partition chromatography. Science, **104,** 84–85.

HIGUCHI, K., AND PETERSON, W. H. 1947 Estimation of types of penicillin in broths and finished products. A microbiological method. Ind. Eng. Chem., Anal. Ed., **19,** 68–71.

HOBBY, G. L., BURKHARDT, B., AND HYMAN, B. 1946 Chemotherapeutic action of various forms of penicillin on hemolytic streptococcal infections in mice. Proc. Soc. Exptl. Biol. Med., **63,** 296-301.

LIBBY, R. L., AND HOLMBERG, N. L. 1945 The activity of penicillins G and X *in vitro*. Science, **102,** 303-304.

SCHMIDT, W. H., WARD, G. E., AND COGHILL, R. D. 1945 Penicillin. VI. Effect of dissociation phases of *Bacillus subtilis* on penicillin assay. J. Bact., **49,** 411–412.

VELDEE, M. V., HERWICK, R. P., AND COGHILL, R. D. 1945 Recommendations of the International Conference on Penicillin. Science, **101,** 42–43.

WELCH, H., PUTNAM, L. E., RANDALL, W. A., AND HERWICK, R. P. 1944 Penicillin. X. Successful treatment of gonorrhea with a single intramuscular injection. J. Am. Med. Assoc., **126,** 1024.