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A Test for Penicillin Sensitivity and Resistance in Staphylococcus.*

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The occurrence of penicillin-fast strains of bacterial species generally susceptible to the action of penicillin has been recorded repeatedly.[1] It has been established that a susceptible strain may acquire penicillin resistance *in vivo* following penicillin treatment, or *in vitro* after growth in penicillin-containing media. The mechanism of acquisition of penicillin fastness *in vitro* by staphylococci was studied by Demerec[2] who found that even very susceptible strains of staphylococci give origin to a few variants resistant to small concentrations of penicillin. The variants in turn, once allowed to grow, give variants of higher resistance. In several steps, but never in one step, it is possible to obtain variants resistant to very high concentrations, up to 250 or more units per ml. This type of resistance is generally permanent. Using the statistical method devised by Luria and Delbruck[3] for the study of bacteriophage resistance, Demerec was able to show that these penicillin-fast variants arise by rare mutations from sensitive organisms, independently of the action of penicillin itself. In all probability the drug merely acts as a selective agent which, by inhibiting the growth of sensitive individuals, allows the mutant individuals of sufficient resistance to grow. Resistance to high concentrations is the result of successive mutations, each contributing further resistance.

The situation is complicated by the occurrence of another type of penicillin resistance in staphylococci.[1] Many of the so-called resistant strains isolated from patients, either before or after treatment with penicillin, seem to owe their ability to withstand the antibiotic to the production of a penicillin inactivator, probably of enzymatic nature (penicillinase[4]). Experiments discussed in this paper show that penicillinase production need not be associated with penicillin resistance of the individual cells.

One important requirement in experimental work and in hospital laboratory routine is the availability of a reliable test for penicillin resistance in staphylococcus. The test should indicate the degree of sensitivity of the bulk of a bacterial population and the presence and proportion in it of cells of higher resistance. For penicillinase-producing strains, the test should indicate the degree of sensitivity of individual cells. Such a test is described in the present paper.

Technic. For the experiments on survival in agar containing penicillin pour plates were used. These were made by introducing the desired amount of penicillin, and then a measured bacterial inoculum, into a tube containing 10 ml of melted nutrient agar (at 45°C), mixing the contents of the tube thoroughly, and plating immediately. With this method, no heat inactivation of penicillin takes place. Results were read after 72 hours of incubation at 37°C.

Penicillin was kept in a refrigerator in

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[1] See Spink, W. W., Hall, W. H., and Ferris, V., *J. A. M. A.*, 1945, **128**, 555.

[2] Demerec, M., *Proc. Nat. Acad. Sc.*, 1945, **31**, 16.

[3] Luria, S. E., and Delbruck, M., *Genetics*, 1943, **28**, 491.

[4] Abraham, E. P., and Chain, E., *Nature*, 1940, **146**, 837.

TABLE I.
Survival of Penicillin sensitive Staphylococci in Agar Containing Penicillin.

Penicillin, units/ml	Survival in agar by colony counts: Strain NRRL, bacteria/ml	Strain 02, bacteria/ml	Strain 5399, bacteria/ml
0.00	2.2×10^8	2.4×10^8	2.7×10^8
0.01	2.5×10^6		
0.02	2.5×10^4	5.0×10^3	2.0×10^4
0.03	1.0×10^4	4.5×10^2	9.0×10^2
0.04	4.0×10^3	5×10^1	3.0×10^1
0.06	1.3×10^2	6×10^0	
0.08	3.0×10^1	0	1.0×10^1
0.10	0	0	0

TABLE II.
Survival of Staphylococci Made Resistant to Penicillin.

		Penicillin, units/ml	Survival by colony count, bacteria/ml
a. Strain from a colony grown in presence of	0.06 units/ml	0.00	5×10^8
		0.04	3×10^8
		0.08	5×10^5
		0.12	2×10^4
		0.16	4×10^3
		0.24	2×10^2
		0.32	0
b. *idem*	0.4 "	0.0	7×10^8
		0.2	9×10^6
		0.3	3×10^5
		0.4	1×10^5
		0.5	2×10^3
		0.75	2×10^2
		1.00	0
c. *idem*	1.0 "	0.0	6×10^8
		1.0	4×10^4
		2.0	1×10^4
		4.0	1×10^3
		6.0	2×10^1
		8.0	1×10^1
		12.0	0

stock solutions of 10,000 units per ml in phosphate buffer of pH 6.9. The stock was titrated at frequent intervals by the cup method,[5] and compared with a penicillin standard. A stock was discarded as soon as its titer began to diminish appreciably.

Tests for penicillinase activity were made by mixing 1 ml of the sample to be tested with 1 ml of a penicillin solution of the desired concentration (generally 40 units/ml). The mixtures were incubated at 37°C for 1 or 2 hours, then assayed, directly or after dilution, by the cup method. Acetone-ether dried preparations for penicillinase tests were made according to Harper.[6]

Results. When sensitive strains of staphylococci are plated in agar containing penicillin, the results are of the type illustrated in Table I.

If bacteria from any of the colonies grown in the presence of the largest amount of penicillin in the experiments of Table I are picked out, grown in broth, and retested in penicillin agar, results of the type illustrated in Table II, a, are obtained. By sampling out and testing colonies grown in the presence of progressively increasing amounts of

[5] Schmidt, W. H., and Moyer, A. J., *J. Bact.*, 1944, **47**, 199.

[6] Harper, G. J., *Lancet*, 1943, **245**, 569.

TABLE III.
Survival of Penicillinase-producing Staphylococci in Agar Containing Penicillin.

	Growth in penicillin agar		
	Penicillin	Inoculum, per plate	
		5×10^7	5×10^5
	Units/ml	Colony count*	Colony count*
a. *Staphylococcus albus* Shaeffer	0.00	Continuous growth	5×10^5
	0.50	"	10^4
	1.00	"	2×10^3
	2.00	"	3×10^2
	5.00	"	0
	10.00	10^5	0
b. *Staphylococcus albus* Cleaves	0.00	Continuous growth	5×10^5
	0.05	"	10^4
	0.20	"	1×10^3
	0.50	10^5	5×10^1
	1.00	10^4	0
	2.00	1.9×10^1	0

* Continuous growth corresponds to more than 1×10^6 colonies per plate. Counts higher than 1×10^3 are estimates.

TABLE IV.
Growth of Sensitive Staphylococci in Agar and Broth Containing Penicillin.

	Platings in agar		Inoculations in broth Inoculum, bacteria per tube									
	Survival by		2.8×10^7		2.8×10^6		2.8×10^5		2.8×10^4		2.8×10^3	
Penicillin, units/ml	colony counts bacteria/ml	Survival ratio	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
0.00	2.8×10^8	1	+	+	+	+	+	+	+	+	+	+
0.01	1.0×10^6	3.5×10^{-2}	+	+	+	+	+	+	+	+	+	+
0.02	5.0×10^4	1.8×10^{-4}	+	+	+	+	+	+	+	—	—	—
0.04	2.0×10^2	7.1×10^{-7}	+	+	+	+	—	—	—	—	—	—
0.06	1.3×10^2	4.6×10^{-7}	+	+	±	+	—	—	—	—	—	—
0.08	3.0×10^1	1.1×10^{-7}	+	+	—	—	—	—	—	—	—	—
0.10	5×10^0	2×10^{-8}	—	—	—	—	—	—	—	—	—	—

(a) = predicted; (b) = experimental; + = visible growth; — = no visible growth.

penicillin, results are finally obtained like those shown in Table II, b and c. The strain of Table II, c, appears to be already quite resistant. These results fully confirm those of Demerec.[2]

The data in Tables I and II were obtained by plating suitable numbers of bacteria in agar containing penicillin; in these cases, plates with the same concentration of penicillin and with different inocula give counts proportional to the size of the inoculum. When some of the "resistant" strains isolated from patients are tested, however, the results are often of the type illustrated in Table III. Here the survival, given by colony counts for each concentration of penicillin, is not proportional to the size of the inoculum, but is much higher for heavy inocula.

Each one of the strains giving results of this latter type was found by proper tests to produce penicillinase. Penicillin inactivation was effected both by the whole bacterial cultures and by acetone-ether dried preparations according to Harper.

Results like those recorded in Table III show that the protection afforded by penicillinase is a mass phenomenon, occurring only when bacteria are inoculated in large number. The initial presence of many individuals is probably needed to yield protective amounts of penicillinase. The amounts of extracellular penicillinase carried over with a heavy inoculum are not responsible for the

protection, as proved by experiments in which the inoculum consisted of thrice washed cells: the results were similar to those with the whole culture. Moreover, if one uses a small inoculum suspended in the clear centrifugate of a fully grown culture, the amount of survival is not appreciably higher.

It is worth mentioning, incidentally, that in our work we found very little penicillinase activity in the supernatant of penicillinase-producing cultures, and no activity was recovered by filtration through sintered glass, Seitz, or Mandler filters, not even after eluting the filters with phosphate buffer.

An important conclusion from experiments like those represented in Table III is that, when penicillin resistance is of the "penicillinase" type, the individual cells may be much more sensitive than the strain appears to be when tested as a whole. In cases like that illustrated by Table III, b, the individual organisms are almost as sensitive as the cells of a sensitive strain.

Platings in penicillin agar, like those described in Tables I-III, represent a good test for penicillin resistance. In practice, however, the method of quantitative platings is not convenient because of its technical complexity. It was therefore desirable to find out if corresponding results could be obtained in liquid medium.

For bacterial strains that do not produce penicillinase, we may expect that, if the bacteria are inoculated into liquid medium containing various concentrations of penicillin, visible growth will appear whenever the inoculum contains at least one individual resistant to the concentration of penicillin present. By using different inoculum-sizes, it should be possible to estimate the proportion of resistant individuals.

For bacterial strains that produce penicillinase, we may expect visible growth whenever enough penicillinase is produced to destroy the amount of penicillin present. If most cells of the strain are sensitive there should be a large discrepancy between tubes with large and with small inocula. In all cases, therefore, *important inoculum-size effects are to be expected.*

These expectations were fully confirmed by experiments. Table IV shows the results of a typical experiment with a sensitive strain, in which different amounts of bacteria from the same culture were introduced into penicillin broth and into penicillin agar. From the colony counts in agar the expectation of growth in each tube was predicted (columns a) and compared with experimental results (columns b). Predicted and actual results coincide satisfactorily.

Similar results were obtained with all strains not producing penicillinase, whatever their degree of resistance. Visible growth in broth appears whenever the inoculum contains at least one individual resistant to the concentration of penicillin employed.

In similar tests for the strains producing penicillinase, the tubes with heavy inoculum showed growth even with very high concentrations of penicillin; the tubes with small inoculum did or did not show growth depending on the sensitivity of the individual cells, as indicated by independent measurements of survival in agar (Table III).

For the purpose of practical measurement of penicillin resistance, we recommend the following test, which can easily be performed in hospital laboratories and whose results can be read within 24 hours.

A good liquid medium (brain-heart infusion, tryptose broth) is tubed in 5 ml amounts, autoclaved, and stored in the cold. For each test, the tubes are numbered as indicated in Table V. Penicillin is diluted in phosphate buffer before the test to concentration of 5 units per ml (Solution I) and the required amounts are added to the tubes with a 1 ml pipette.

From the culture to be tested, well-grown but not older than 48 hours, 2 successive dilutions (1:100 and 1:10,000) are prepared in 2 tubes of the same medium used for the test (0.05 ml into 5 ml). 0.1 ml of the original culture, or of one of the dilutions, is then introduced into each tube as indicated in the table. For well-grown cultures, the inocula from the undiluted culture and from the two dilutions correspond respectively to 5×10^7, 5×10^5, and 5×10^3 organisms. The tubes are incubated and the results read

TABLE V.
Test for Penicillin Resistance.

Penicillin			a. *Staphylococcus aureus* NRRL Inoculum						b. *Staphylococcus aureus* 26 Inoculum					
Units/ml	Units/tube	Amts of Solution I ml/tube	0.1 ml of culture Tube No.		0.1 ml of dilution 1:100 Tube No.		0.1 ml of dilution 1:10,000 Tube No.		0.1 ml of culture Tube No.		0.1 ml of dilution 1:100 Tube No.		0.1 ml of dilution 1:10,000 Tube No.	
0.00	0.00	0	1	+	2	+	3	+	1	+	2	+	3	+
0.05	0.25	0.05*	4	+	5	—	6	—	4	+	5	+	6	+
0.20	1.00	0.20	7	—	8	—	9	—	7	+	8	+	9	—
0.50	2.50	0.50	10	—	11	—	12	—	10	+	11	+	12	—

* = 1 drop from a 1 ml pipette.

TABLE VI.
Test for Penicillin Resistance.

	Penicillin	Inoculum					
		0.1 ml of culture		0.1 ml of dilution 1:100		0.1 ml of dilution 1:10,000	
	units/ml	Tube No.		Tube No.		Tube No.	
a. *Staphylococcus albus* Shaeffer	0.00	1	+	2	+	3	+
	0.05	4	+	5	+	6	+
	0.20	7	+	8	+	9	+
	0.50	10	+	11	+	12	+
	2.00	13	+	14	±	15	±
	5.00	16	+	17	—	18	—
b. *Staphylococcus albus* Cleaves	0.00	1	+	2	+	3	+
	0.05	4	+	5	+	6	±
	0.20	7	+	8	—	9	—
	0.50	10	+	11	—	12	—
	2.00	13	+	14	—	15	—
	5.00	16	+	17	—	18	—

after 24 hours; a further reading after 48 hours may sometimes show some more positive results.

Table V, a, shows the results for a typical sensitive strain; Table V, b, for a strain of medium resistance; Table VI for two penicillinase-producing strains. For resistant strains we generally repeat the test using amounts of penicillin ten times larger, as shown in Table VI. The amounts of resistant and sensitive cells in each strain are immediately evident by an inspection of the tables.

Discussion. The results described in this paper indicate a definite influence of the size of the inoculum in penicillin sensitivity tests for staphylococcus. The importance of inoculum-size effects for penicillin activity has generally been belittled in the literature on the basis of some early observations, mainly concerned with gross differences in sensitivity.[7]

The inoculum-size effects depends, first, on the presence in sensitive strains of a few mutant individuals of higher resistance; second, on the occurrence of strains producing penicillinase but consisting of cells individually sensitive.

The origin of the penicillinase-producing strains still awaits clarification. Since strains producing large amounts of penicillinase may consist of cells individually sensitive to penicillin, it seems unlikely that penicillinase production be secondarily acquired by resistant strains when grown in presence of penicillin.

[7] Abraham, E. P., Chain, E., Fletcher, C. M., Gardner, A. D., Heatley, N. G., Jennings, M. A., and Florey, H. W., *Lancet*, 1941, **241**, 177.

The current tests for penicillin sensitivity[8] do not take into account the inoculum size effects. The use of paper strip tests on heavily inoculated plates is likely to be misleading in cases of relatively sensitive strains producing penicillinase.

A simple test for penicillin resistance is proposed, which indicates the sensitivity of a staphylococcus culture, the presence and proportion of resistant individuals in it, and the degree of their resistance. The test also distinguishes resistant strains from relatively sensitive strains that produce penicillinase. This test may appear to be too delicate because of the fine differences in penicillin sensitivity which it reveals. It must be kept in mind, however, that concentrations of the order of 0.5 units per ml are almost the highest that can be obtained in the blood of patients. A reduction in blood level from 0.5 to 0.2 units per ml in the course of treatment may give a chance for growth to resistant bacterial cells present in a relatively sensitive strain. Moreover, the stepwise increase in bacterial resistance should lead us to consider any strain that is not very sensitive as a potential source of highly resistant variants. The therapeutic use of insufficient concentrations of penicillin may allow these variants to grow and spread, thus nullifying the usefulness of penicillin as a therapeutic agent.

Infections caused by penicillinase-producing strains consisting of individually sensitive cells may be susceptible to penicillin therapy if the number of bacteria present is small enough so that little penicillinase production occurs.

The use of similar tests for bacteria other than staphylococci should await more evidence regarding penicillin-fastness in those organisms.

Summary. The results of penicillin sensitivity tests for staphylococci are influenced by the size of the inoculum on two accounts: (a) the presence in sensitive strains of a minority of resistant individuals originating by mutation; and (b) the occurrence of penicillinase-producing strains whose cells are individually sensitive to penicillin. A simple laboratory test is proposed for the detection and quantitative measurement of penicillin resistance in staphylococcus.

[8] Rammelkamp, C. H., and Maxon, T., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 386; Spink, W. W., and Ferris, V., *Science*, 1945, **102**, 221.

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Effect of Anesthetics and Convulsants on Brain Acetylcholine Content.

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Whether acetylcholine plays an important role in central nervous system synaptic transmission has long been a matter of interest. It is known that central neural tissue contains the ester, can respond to its administration and can synthesize it. Though such facts, taken together with information on peripheral conduction, may suggest a transmitter role for acetylcholine in the central nervous system they do not prove it. Therefore, additional evidence has been sought, and one approach has been to attempt a correlation of acetylcholine content with activity. Results have been varied. Thus, stimulation of afferent nerves has been found to increase production or liberation of acetylcholine by brain,[1,2] but negative results have also been reported.[3,4] Direct faradization

[1] Cortell, R., Feldman, J., and Gellhorn, E., *Am. J. Physiol.*, 1941, **132**, 588.

[2] Dikshit, B. B., *J. Physiol.*, 1934, **80**, 409.

[3] Adam, H. M., McKail, R. A., Obrador, S., and Wilson, W. C., *J. Physiol.*, 1938, **93**, 45P.

[4] Feldberg, W., and Schriever, H., *J. Physiol.*, 1936, **86**, 277.