

THE MULTIPLE MECHANISMS OF PENICILLIN RESISTANCE

HARRY EAGLE

WITH THE TECHNICAL ASSISTANCE OF MINA LEVY AND RALPH FLEISCHMAN

Section on Experimental Therapeutics, Laboratory of Infectious Diseases, National Microbiological Institute, National Institutes of Health,¹ Bethesda, Maryland

Received for publication May 17, 1954

With a number of bacterial strains, resistance to penicillin is related to the fact that the bacteria release into the surrounding medium an enzyme, penicillinase, which converts penicillin to penicilloic acid. If the bacterial population is sufficiently large, the antibiotic may thereby be inactivated before it can react with the cell. In addition, a few resistant strains have been shown to be actually penicillin dependent (Barber, 1953). However, these two factors of penicillin inactivation and penicillin dependence account for the resistance of only a limited number of bacterial species and strains.

The use of S³⁵-labeled penicillin has permitted an analysis of the reaction between bacteria and the antibiotic in terms of the amount bound, the amount of radioactive material which can be eluted from the penicillin treated cells, and the antibacterial activity of the eluate. On the basis of such studies, at least three additional types of penicillin resistant bacteria may now be distinguished, and several mechanisms making for resistance may be operative in the same cell.

MATERIALS AND METHODS

The characteristics of the S³⁵-labeled penicillin and the methods used in assaying the amounts bound by intact bacteria and by cell-free sonic extracts have been described in a previous communication (Eagle, 1954a). In the present experiments also, bacteria in the logarithmic phase of growth were exposed to radioactive penicillin for 2 hr at 37 C and washed twice prior to the determination of bound "penicillin". For the elution experiments (table 1), the penicillin treated bacteria were sedimented, the supernatant withdrawn, and the sediment incubated for varying periods at 37 C preliminary to extraction with 10 volumes of broth for 1 hr at 37 C. The radioactivity of the eluate served as a measure

of its total "penicillin" content and was compared with its antibacterial activity as determined by bioassay with *Streptococcus pyogenes*. In the experiments to determine the inactivation of penicillin by sonic extracts (table 1), the cell-free material was incubated with penicillin for 1 hr at 37 C and then centrifuged in the Spinco centrifuge for 4 hr at 40,000 rpm (68,000–144,000 G). The radioactivity and antibacterial activity of the supernatant were determined after sterilization by filtration through sintered glass. When the radioactivity of the final product was too low to permit dilution, self-absorption was controlled by adding a known amount of radio-penicillin (e.g., 5×10^{-3} μ g) to the sample to be measured. The difference between the counts with and without added penicillin then served as a measure of the counts per 5×10^{-3} μ g of penicillin in the sample.

The courtesy of Drs. W. A. Randall and H. Welch of the Food and Drug Administration, in supplying many of the strains used in the present study, is gratefully acknowledged.

EXPERIMENTAL RESULTS

(1) *Intracellular degradation of penicillin*. A number of resistant bacteria which do not liberate significant amounts of penicillinase into the medium can nevertheless inactivate penicillin after it has entered the cell. This was shown (Eagle, 1954a) to be the case with the K12 strain of *Escherichia coli* and has since been observed with a strain of *Shigella*, two strains of *Proteus morganii*, and an additional strain of *E. coli* (bottom portion of table 1). None of these strains liberated significant amounts of penicillinase into the medium. When penicillin at 0.1 μ g per ml was added to fully grown cultures, there was only a partial degradation of the antibiotic after 3 to 5 hr at 37 C. However, when these strains were exposed to radioactive penicillin, and the sedimented organisms were then eluted with

¹ Public Health Service, U. S. Department of Health, Education, and Welfare.

TABLE 1

Showing that, except for a few highly resistant strains (bottom section of table), variations in penicillin resistance are not related to the ability of the bacteria to degrade the antibiotic

Bacterial Species Tested	LD ₅₀	(a)			(b)	
		Antibacterial Activity of "Penicillin" in Eluate of Penicillin Treated Bacteria			Residual Penicillin Activity* after Incubation of Antibiotic with Cell-free Bacterial Extracts for 1 hr at 37 C	
		Cells exposed to 1 µg/ml, sedimented, and incubated at 37 C prior to elution		Cells exposed to 100 µg/ml, sedimented, and eluted immediately	0.1 µg/ml	100 µg/ml
		1 hr	4 hr			
	µg/ml	%	%		%	%
<i>S. pyogenes</i>	0.006	29	0	72	30	>95
<i>C. pseudodiphtheriae</i> (302)†	0.007	67	—	—	73‡	68‡
<i>D. pneumoniae</i>	0.016	60	49	81	73	—
<i>M. pyogenes</i> (Smith)	0.024	72	30	>95	27	>95
<i>C. porcine</i> (311)†	0.15	58	—	>95	24‡	55‡
<i>C. pyogenes</i> (307)	0.35	100	—	83	65	84
<i>S. faecalis</i>	2	44	18	>95	59	>95
<i>M. pyogenes</i> (138)	2.5	41	22	62	68	>95
<i>S. pullorum</i> (903)	2.8	24	—	>95	71	89
<i>Proteus</i> sp. (704)	6.2	33	—	56	87	>95
<i>P. ammoniae</i> (714)	16	33	—	60, 54	55	>95
<i>S. oregon</i> (909)	22	35	—	>95	71	80
<i>S. paradysenteriae</i> (959)	58	39	—	0, 0	—	0
<i>E. coli</i> (B)	10	0	0	0	0	0
<i>E. coli</i> (K12)	30	0	0	0	0	0
<i>P. morganii</i> (707)	160	0	0	0	0	0
<i>P. morganii</i> (710)	510	0	0	0	0	0

* Antibacterial activity was determined by bioassay with *Streptococcus pyogenes* and is referred in the table to the total "penicillin" content of the eluate (a) (or supernatant (b)), as determined by radioactivity.

† F. D. code numbers.

‡ Sonic extracts incubated with penicillin for 2 hours.

broth, radioactive material was extracted which was devoid of bactericidal action. Even after exposure to 100 µg per ml, when the diffusible intracellular penicillin varied between 50 and 75 µg per ml bacteria, that intracellular material was completely degraded in the time required to sediment the bacteria and to add the eluting fluid. It is a reasonable presumption that the ability of these bacterial strains to degrade penicillin may be largely responsible for their relative resistance to the antibiotic. In contrast, with organisms which did not have this capacity to degrade penicillin intracellularly, actively bactericidal penicillin could be extracted from the cell even after exposure to only 1 µg per ml and after the sedimented cells had been in-

culated for as long as 4 hr prior to elution (table 1).²

With the 5 strains which rapidly inactivated free intracellular penicillin, cell-free bacterial extracts prepared by sonic vibration were similarly active (table 1). Such extracts at concentrations of 20 mg per ml completely inactivated 100 µg per ml penicillin in 1 hr. With the remaining

* In interpreting the apparent partial inactivation of penicillin by these strains, it should be borne in mind that the eluate contains not only the freely diffusible unbound intracellular penicillin but also penicillin which had combined with cellular components, and which was partially dissociated on washing (cf Eagle, 1954a). That bound penicillin may have been chemically altered and biologically inactivated by its combination.

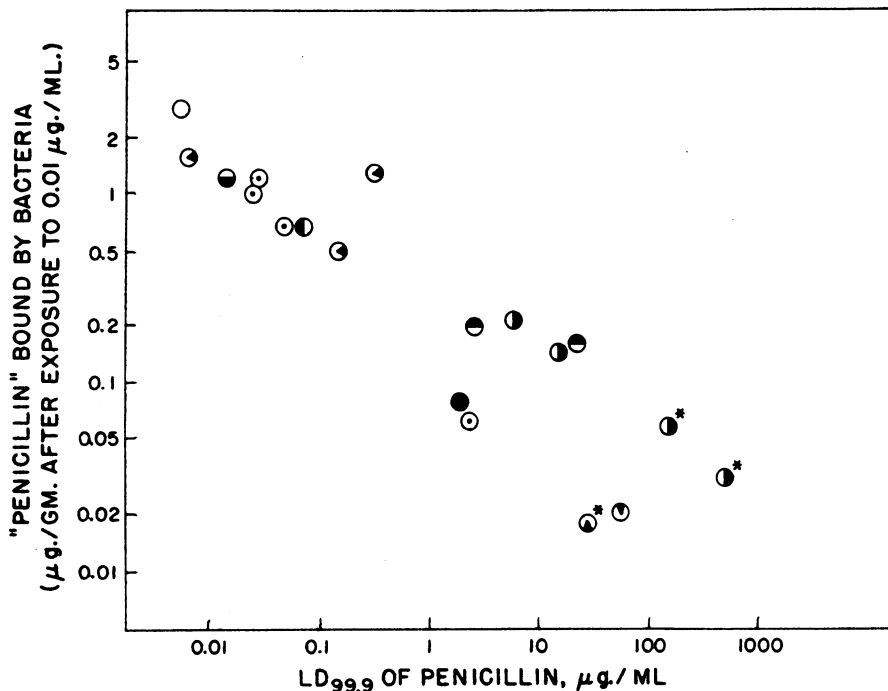


Figure 1. The amounts of isotopically labeled "penicillin" bound by washed bacteria after exposure to 0.01 $\mu\text{g}/\text{ml}$, considered as a function of their sensitivity to the antibiotic. Bacteria in the logarithmic phase of growth were exposed to the S^{35} -labeled penicillin for 2 hr at 37 C and washed twice prior to the determination of bound "penicillin" by radioactivity measurement. The points indicated with an asterisk indicate bacterial strains which rapidly degraded the free intracellular penicillin to an inactive form.

- | | |
|---------------------------------|------------------------------|
| ○ <i>Streptococcus pyogenes</i> | ● <i>Salmonella sp.</i> |
| ⊙ <i>Micrococcus pyogenes</i> | ⊙ <i>Shigella sp.</i> |
| ● <i>Diplococcus pneumoniae</i> | ⊙ <i>Corynebacterium sp.</i> |
| ● <i>Streptococcus faecalis</i> | ⊙ <i>Escherichia coli</i> |
| ● <i>Micrococcus flavus</i> | ⊙ <i>Bacillus sp.</i> |
| ● <i>Proteus sp.</i> | |

strains, which inactivated free intracellular antibiotic to only a minor degree, sonic extracts were usually also relatively inactive. As much as 20 mg extract per ml, added to penicillin at 0.1 μg per ml, caused only partial inactivation in 1 hr at 37 C.

(2) *Low reactivity of the cell with penicillin.* The ability of some bacterial strains to destroy penicillin, either by liberating penicillinase into the medium or by degrading the antibiotic which does get into the cell, is, however, not a general explanation of "natural" penicillin resistance. Bacteria with no significant ability to inactivate the antibiotics nevertheless varied widely in their penicillin sensitivity. In the strains shown in the upper portion of table 1 and in figure 1, the LD_{99.9}

varied from 0.006 μg per ml to 2.5 μg per ml. None of these either produced penicillinase or had a significant effect on the unbound penicillin within the cell. The explanation of this "natural" variation in penicillin sensitivity may lie in the recent demonstration (Rowley *et al.*, 1950; Eagle, 1954a) that the amounts of penicillin bound under standardized conditions by *Micrococcus pyogenes*, *Klebsiella pneumoniae*, *Escherichia coli*, *Streptococcus pyogenes*, *Diplococcus pneumoniae*, and *Streptococcus faecalis* varied directly with their penicillin sensitivity. A larger number of bacterial species and strains have now been studied in this respect, with the results summarized in figure 1. The high degree of correlation between the degree to which the antibi-

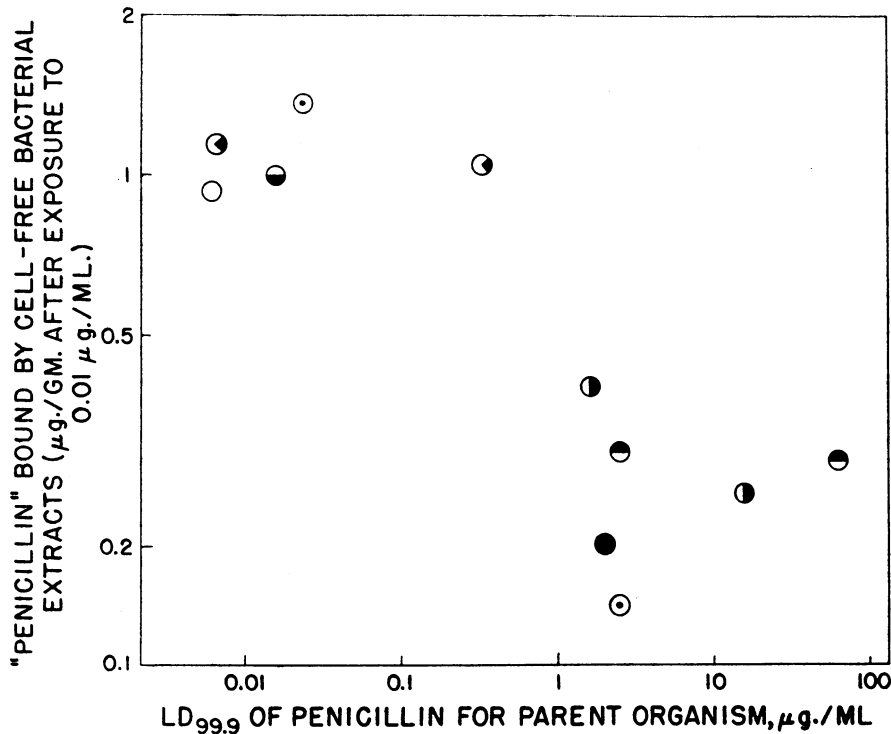


Figure 2. The amounts of penicillin bound by cell-free sonic extracts of various bacterial species after exposure to 0.01 $\mu\text{g./ml}$, considered as a function of the penicillin sensitivity of the parent organisms.

Cell-free sonic extracts (Eagle, 1954a) were incubated with S^{35} -labeled penicillin at 0.01 $\mu\text{g./ml}$ for 2 hr at 37 C. The large molecular weight components were then sedimented in the ultracentrifuge at 68,000-144,000 G, the sedimented material was redissolved in water, and resedimented prior to the determination of its "penicillin" content by radioactivity measurement. Sonic extracts which degraded penicillin (*cf* text) are not included in the figure. The key to the identification of the strains is given in the legend to figure 1.

otic was bound and the penicillin sensitivity of the cell is self-evident. These differences in penicillin uptake are not referable to corresponding differences in the permeability of the cells to the antibiotic. As previously found in a limited number of strains and confirmed for the strains here studied, cell-free sonic extracts bound penicillin in roughly the same order as the intact cells and again in relation to their sensitivity (figure 2).

The working hypothesis has therefore been suggested (Eagle, 1954a) that the widely varying penicillin sensitivity of bacteria in nature is related to, and probably determined by, the correspondingly varying reactivity with penicillin of cell components which are vital to the cellular economy and which are inactivated by their combination with the antibiotic. In the sensitive cell, these penicillin vulnerable components are

highly reactive, and a small amount of penicillin suffices to bring about their inactivation. With the relatively insensitive cell, however, the penicillin vulnerable components are less reactive, and correspondingly higher concentrations must be added to the medium in order to effect the necessary degree of combination. In both cases, the over-all reactivity of the cell with penicillin apparently parallels the reactivity of the penicillin vulnerable component. It is significant in this connection that on the basis of indirect but none the less cogent evidence, Davis and Maas (1952) concluded that the development of resistance of *E. coli* to sulfathiazole and to *p*-nitrobenzoic acid was related to a decreased combining affinity of a cellular enzyme for the inhibitor, relative to its affinity for the normal substrate. Similarly, the relative nontoxicity of penicillin for mammalian cells is not due to the failure of

TABLE 2

The relative constancy of the amounts of penicillin bound at the lethal level ($LD_{99.9}$) by organisms in the sensitivity range 0.006–2.5 $\mu\text{g/ml}$

With five strains, the amounts bound at the $LD_{99.9}$ concentration were obtained by graphic interpolation (Eagle, 1954a); these interpolated values are shown as italics in the table. With the remaining 12 the organisms were tested directly at the $LD_{99.9}$ concentrations as determined in agar. None of the strains here listed produced extracellular penicillinase. Four highly resistant strains (*E. coli*, *P. morganii*, *S. paradysenteriae*) did rapidly degrade intracellular penicillin.

Bacterial Species Tested	$LD_{99.9}$	"Penicillin" Bound by Bacteria after Exposure for 2 hr at 37 C to $LD_{99.9}$ Concentrations and Retained after Washing	
		$\mu\text{g/g}$ dry weight	molecules/cell
<i>S. pyogenes</i>	0.006	<i>2.5</i>	<i>3,300</i>
<i>C. pseudodiphtheriae</i> (302)†	0.007	1.65	3,300±*
<i>D. pneumoniae</i>	0.016	<i>1.8</i>	<i>1,600</i>
<i>M. pyogenes</i> (Smith)	0.024	<i>1.7</i>	<i>2,000</i>
<i>M. pyogenes</i> (F 12)	0.05	2.6	3,150
<i>C. porcine</i> (311)	0.15	1.27	2,700±*
<i>C. pyogenes</i> (307)	0.35	1.77	—
<i>S. faecalis</i>	2	4	<i>2,400</i>
<i>M. pyogenes</i> (138)	2.5	2.6	3,150
<i>S. pullorum</i> (903)	2.8	3.16	5,400
<i>Proteus</i> sp. (704)	6.2	2.35	7,900
<i>P. ammoniae</i> (714)	16	5.0	10,200
<i>S. oregon</i> (909)	22	11.5	16,300
<i>E. coli</i> (K12)	30	10.3	21,000
<i>S. paradysenteriae</i> (959)	58	70	54,600
<i>P. morganii</i> (707)	160	118	134,000
<i>P. morganii</i> (710)	510	337	623,000

* Approximations only, because of difficulty in counting the clumped bacteria.

† F. D. code numbers.

the antibiotic to get into the cell, but is probably related to the low combining affinity of cell components with the intracellular penicillin (Eagle, 1954c).

If the combining affinity of a vital cell component with a drug is in fact a determinant of sensitivity to its cytotoxic action, as originally suggested by Ehrlich, it follows that a chemotherapeutic agent may have a selective toxicity for the invading microorganism even when the

parasite and the host cells have similar metabolic requirements and similar metabolic pathways, and even when they are equally permeable to the drug.

It is of interest that at the lethal concentration of penicillin, comparable amounts of the antibiotic were bound by both sensitive and resistant strains. The lethal concentration of penicillin thus appears to be the amount which must be present in the surrounding fluid in order to effect the uniform degree of binding which renders the cells nonviable. Seventeen strains have now been studied (table 2 and figure 3) in which the lethal concentration of penicillin ($LD_{99.9}$) varied 400-fold, from 0.006 to 2.5 μg per ml. The amount of penicillin bound at these $LD_{99.9}$ levels varied only from 1.65 to 4 μg per g dry weight, and from 1,600 to 3,300 molecules per cell. (The much larger concentrations bound by highly resistant organisms, which were killed only by concentrations in excess of e.g. 5 μg per ml (right-hand portion of figure 3), are not relevant to this discussion. At these high concentrations, penicillin and its degradation products alike are nonspecifically bound by sensitive and resistant organisms alike (Eagle, 1954a), and this nonspecific binding probably has no bearing on the mode of action of the antibiotic.)

It is an open question whether the over-all reactivity of the cell with penicillin, which parallels their sensitivity, is determined largely by a few penicillin vulnerable components, or whether instead a large number of cellular components are reactive, only a few of which are vital to the cell and inactivated by their combination. Under such circumstances, although 1,600 to 3,300 molecules have combined with the cell at the lethal concentration of penicillin, a much smaller number may be in combination with the vital components, the inactivation of which is responsible for the death of the cell.

(3) *Unknown mechanisms of resistance (penicillin resistant variants of normally sensitive bacteria)*. As discussed in the preceding sections, with bacteria as isolated in nature, penicillin sensitivity has regularly been related to the amount bound by the cell under standardized conditions. Conversely, resistance has regularly been associated with the failure of the cell to combine with penicillin, either because of the production of penicillinase (section 1 above), because of the intracellular degradation of the antibiotic (section 2

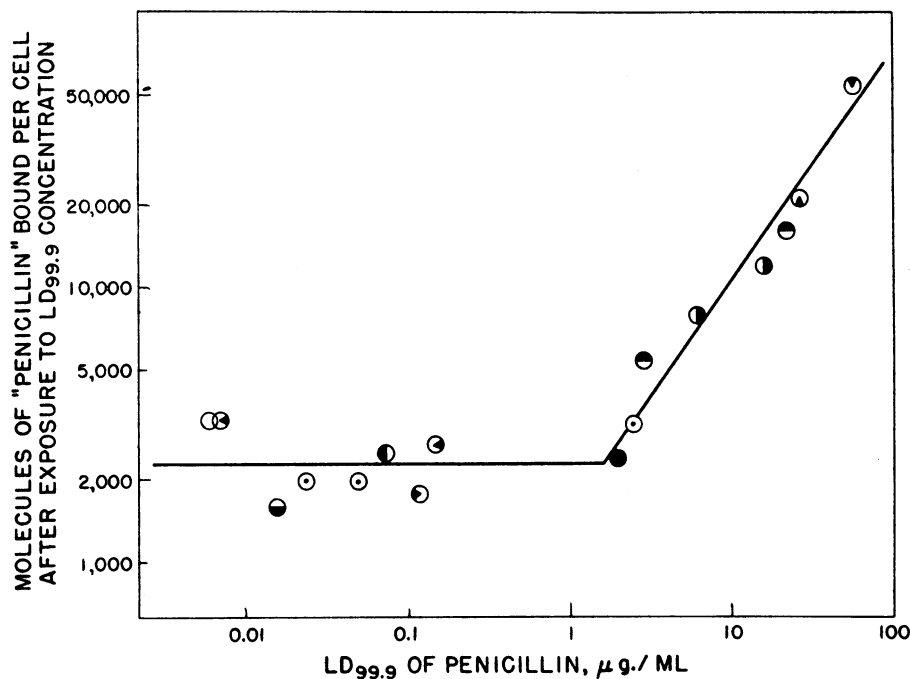


Figure 3. The amounts of S³⁵-labeled "penicillin" bound by 16 bacterial strains at the LD_{99.9} concentration, considered as a function of their penicillin sensitivity.

Each point in the figure represents a different strain (key in figure 1).

above), or because the penicillin vulnerable components of the cell have an inherently low order of reactivity with the antibiotic (section 3 above). However, the foregoing generalization applies only to bacteria as they are isolated in nature. When resistant variants are obtained by the selective propagation of initially sensitive cultures in increasing concentrations of antibiotic, the resistance of those variant strains is no longer related to the reactivity of the cell with antibiotic. Some of the resistant variants do bind significantly less, as would be expected from the data of the preceding section; but some variants are unchanged in their reactivity, and some actually bind more penicillin than the parent sensitive cell (Eagle, 1954b). With these resistant variants, the over-all reactivity of the cell with penicillin is no longer a regular measure of their penicillin sensitivity. If that over-all reactivity of the cell reflects the reactivity of the vulnerable components which are normally inactivated by the antibiotic, resistance in these cases would not be determined by a decreased combining affinity with penicillin, but by other and as yet undefined mechanisms.

The possibility remains, however, that even in these resistant variants the penicillin vulnerable cellular component has become less reactive with the antibiotic. That decreased reactivity could be masked by the continued reactivity of other cellular components which are either not vital to the cell or which are not functionally inactivated by that combination. This and a number of related problems await the isolation and characterization of the penicillin vulnerable component of the cell.

SUMMARY

Some strains of bacteria produce an extracellular penicillinase which converts penicillin to penicilloic acid. The latter is not bactericidal and is not selectively bound by penicillin sensitive bacteria. In addition, Barber (1953) has demonstrated that some resistant strains of *Micrococcus pyogenes* are actually penicillin dependent. However, these two mechanisms account for the resistance of only a limited number of bacterial species and strains.

The experiments reported in this paper using isotopically labeled penicillin indicate at least

three additional mechanisms of penicillin resistance.

(1) A number of strains (e.g., *Escherichia coli*, *Proteus morganii*, and *Shigella paradysenteriae* in the present series) do not liberate penicillinase into the medium, but rapidly degrade penicillin after it enters the cell.

(2) The most important single determinant of penicillin resistance in bacteria as they occur in nature may be their varying combining affinity with the antibiotic. Among bacteria which do not degrade penicillin, resistant strains regularly bind less penicillin under standard conditions than do sensitive strains; and cell-free extracts also bind penicillin in relation to the sensitivity of the intact cell. A reasonable working hypothesis is that the combining affinity of vulnerable cell components with the antibiotic is the actual determinant of penicillin sensitivity. The overall combining affinity of the cell would then parallel the reactivity of vital cellular components which are inactivated by their combination with the antibiotic.

(3) Penicillin resistant variants of normally sensitive cells may, however, combine with penicillin to the same degree as the parent sensitive cell without being killed as the result of that com-

bination. The mechanisms of resistance in such resistant variants are as yet unknown.

REFERENCES

- BARBER, M. 1953 Penicillin-resistant and penicillin-dependent staphylococcal variants. *J. Gen. Microbiol.*, **8**, 111-115.
- DAVIS, B. D., AND MAAS, W. K. 1952 Analysis of the biochemical mechanism of drug resistance in certain bacterial mutants. *Proc. Natl. Acad. Sci. U. S.*, **38**, 775.
- EAGLE, H. 1954a The binding of penicillin in relation to its cytotoxic action. I. Correlation between the penicillin sensitivity and combining activity of intact bacteria and cell-free extracts. *J. Exptl. Med.*, **99**, 207-226.
- EAGLE, H. 1954b The binding of penicillin in relation to its cytotoxic action. II. The reactivity with penicillin of resistant variants of streptococci, pneumococci and staphylococci. *J. Exptl. Med.*, **100**, 103-115.
- EAGLE, H. 1954c The binding of penicillin in relation to its cytotoxic action. III. The binding of penicillin by mammalian cells in tissue culture (HeLa and L strains). *J. Exptl. Med.*, **100**, 117-124.
- ROWLEY, D., COOPER, P. D., ROBERTS, P. W., AND SMITH, E. L. 1950 The site of action of penicillin. 1. Uptake of penicillin on bacteria. *Biochem. J. (London)*, **46**, 157-161.