THE BINDING OF PENICILLIN IN RELATION TO ITS CYTOTOXIC ACTION

II. The Reactivity with Penicillin of Resistant Variants of Streptococci, Pneumococci, and Staphylococci

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A satisfactory correlation has been found between the sensitivity of a number of bacterial strains to penicillin, and their combining affinity for the antibiotic (1). It has been further shown that cell-free extracts of bacteria also bind penicillin, and again in relation to the sensitivity of the strain. The latter observation suggested that the range of penicillin sensitivity may be determined by the differences in the reactivity of one or more cell components with the antibiotic, and not by permeability considerations. Further, at equieffective ($LD_{99.9}$) concentrations, whether 0.006 or 2 μg. per ml., four of five species studied (*Streptococcus pyogenes*, *Micrococcus pyogenes*, *Streptococcus faecalis*, *Diplococcus pneumoniae*) had bound comparable amounts of antibiotic, averaging 1.7 to 4 μg. per gm., and 1600 to 3300 molecules per cell. With these strains, the lethal concentration of penicillin appeared to be that which had to be present in the outside medium in order to effect the requisite degree of combination with vulnerable cell constituents of differing reactivity.

The fifth culture tested (*Escherichia coli*) was found to inactivate the intracellular penicillin rapidly. Its failure to bind the antibiotic, and its relative refractoriness to its bactericidal action, were probably related in part or in whole to that intracellular inactivation.

On the basis of these considerations, it might be anticipated that when an originally penicillin-sensitive strain had been rendered resistant by serial transfer through increasing concentrations of penicillin, that change from sensitivity to resistance would be accompanied by either a decreased combining affinity with the antibiotic, or an enhanced ability to degrade it. Although Rowley *et al.* (2) had found the former to be the case with two resistant strains of *Micrococcus pyogenes*, inconclusive results were obtained by Maass and Johnson (3). As will be here shown, with the strains studied in this laboratory, the development of resistance did not necessarily stem from either of these factors. Some of the resistant variants bound significantly more penicillin than

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did the parent sensitive strains, some bound significantly less, and in one instance there was a progressive decrease in reactivity with penicillin not associated with increasing resistance. In no case was there a demonstrably enhanced ability to inactivate the free intracellular penicillin.

It would appear therefore that qualitatively different factors underlie the penicillin resistance of bacterial strains as they occur in nature, and the resistance produced in originally sensitive strains by their selective propagation in antibiotic. In the latter resistant variants, the over-all reactivity of the cells with penicillin is no longer a measure of their sensitivity to the antibiotic.

Materials and Methods

The methods used for the preparation of the bacterial suspensions and their sonic extracts, for their exposure to radioactive penicillin, and for the measurement of "penicillin" binding, have been described in the preceding paper of this series (1). The resistant variants were obtained by the serial propagation of organisms which had survived maximum sublethal concentrations of penicillin (*cf.* reference 4.)

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EXPERIMENTAL

A. Penicillin Binding

The $LD_{99.9}$ concentrations of penicillin for the strains of *Streptococcus pyogenes*, *Diplococcus pneumoniae*, *Micrococcus pyogenes*, and *Streptococcus faecalis* used in the present experiments were 0.006, 0.016, 0.024, and 2 μg. per ml., respectively. By serial transfer through increasing concentration of antibiotic, these sensitivities were progressively increased to 0.12, 0.3, 16, and 100 μg. per ml., respectively; *i.e.*, increases of 20-, 19-, 660- and 50-fold. The reactivity with penicillin of these resistant variants, and of their cell-free sonic extracts, was determined by the technique described in a preceding paper (1), using S^{35}-labelled penicillin to determine the degree of combination.

1. Streptococcus faecalis.—As shown in Table I, the highly resistant strain of *Streptococcus faecalis* bound slightly but significantly more penicillin from low concentrations of the antibiotic than did the parent, relatively sensitive cell. Further, cell-free extracts of the resistant strain also proved slightly more reactive with penicillin than similar extracts of the parent strain, over the entire range of concentrations studied.

2. Micrococcus pyogenes.—The affinity of several resistant variants of *Micrococcus pyogenes* for penicillin had no necessary relationship to their enhanced resistance. One resistant variant (strain A in Table II) was just as reactive with penicillin as the parent, sensitive culture. A second variant (strain B of Table III) was even more reactive than the parent culture. On November 19, 1952, after five successive transfers in increasing concentrations of penicillin, the $LD_{99.9}$ level of the latter culture was 0.6 μg. per ml., 25 times greater than the parent strain. At that time, when exposed to penicillin concentrations of

TABLE I

The Increased Affinity for Penicillin of a Resistant Variant of S. faecalis

Bacterial cultures in the logarithmic phase of growth were exposed to S^{35}-labelled penicillin at the indicated concentration for 2 hours at 37°C., and the S^{35} content of the sedimented bacteria measured after two washings. The binding by cell-free sonic extracts was determined after sedimentation in the ultracentrifuge, resolution of the large molecular weight components, and resedimentation at 67,000 to 144,000 *g*. In this and following tables, the radioactivity of the test materials is expressed in terms of its penicillin equivalent. The underlined numbers are the averages of the experimental values.

Test materials		$LD_{99.9}$	Concentration of penicillin added to fluid, μg./ml.					
			0.001	0.01	0.1	1	10	100
			"Penicillin" bound by bacteria (or cell-free sonic extract)					
		mg./ml.		μg./gm.	μg/gm.	μg/gm.	μg/gm.	μg/gm.
Intact organisms	Parent culture	2		0.11	0.54	2.15	4.4	34.7
				0.086	0.517	2.1	3.72	20.2
				0.046				
				0.081	0.53	2.12	4.06	27.3
	Resistant variant	100		0.215	1.15	6.1	12.9	30.4
				0.21	0.81	5.28	12.4	22.4
				0.135		3.73		
				0.108				
				0.054				
				0.18	0.98	5.03	12.65	26.4
Cell-free sonic extracts	Parent culture	2	0.0295	0.226	1.94	4.27	7.22	25
			0.016	0.207	1.92	4.26	6.78	21
				0.157	1.86	3.53	5.37	17
							4.4	17
			0.0228	0.2	1.91	4.02	5.94	20
	Resistant variant	100	0.035	0.344	2.76	8.23	16.6	65
			0.023	0.28	2.56	6.6	16.5	65
				0.262	2.2	6.06	16.5	60
			0.029	0.295	2.51	6.96	16.5	63

0.001, 0.01, and 0.1 μg. per ml., these resistant organisms concentrated the antibiotic 94-, 64-, and 8.7-fold, respectively, in each case slightly more than the parent, sensitive strain. At the 0.01 concentration, the sedimented organisms contained 2.1 μg. per gm., as compared with an average value of 1.3 μg. per gm.

TABLE II

The Maintained Affinity for Penicillin of a Resistant Variant of M. pyogenes

Each number in the table is the average of 2 to 4 experimental determinations. The experimental procedure is described in Table I.

Test materials		$LD_{99.9}$	Concentration of penicillin added to fluid, μg./ml.					
			0.001	0.01	0.1	1	10	100
			"Penicillin" bound by bacteria (or by cell-free sonic extract)					
		μg./ml.	μg./gm.	μg/gm.	μg/gm.	μg/gm.	μg/gm.	μg/gm.
Intact bacteria	Parent strain	0.024	0.166	0.99	2.08	2.55	5.2	41
	Resistant variant A*	0.65	0.217	1.01	1.87	3.82	10.8	
Sonic extracts	Parent strain	0.024	0.087	1.36	2.46	2.84	6.56	46
	Resistant variant A*	0.65	0.064	0.67	2.61	3.5	5.76	31

* Obtained by four successive transfers through increasing concentrations of antibiotic (*cf.* reference 4).

TABLE III

Showing That Resistant Variants of M. pyogenes May Have Either an Increased or Decreased Reactivity with Penicillin

	Date of test	$LD_{99.9}$	"Penicillin" bound by bacteria
		μg./ml.	μg./gm.* dry weight
Parent (sensitive) culture	June 1, 1952 to Dec. 16, 1953	0.024	1.21 1.24 1.51 1.23 1.33 1.63
Resistant variant B	Nov. 19, 1952	0.6	2.1
Resistant variant C	Apr. 9, 1953	16.0	0.53
Resistant variant D	Dec. 16, 1953	28.0	0.30

* "Penicillin" bound after exposure for 2 hours to penicillin at 0.01 μg./ml., and retained after washing.

for the parent culture. Over the following thirteen months, the $LD_{99.9}$ was further increased to 28 μg. per ml. by repeated transfers in antibiotic. This highly resistant variant (strain D in Table III) now concentrated the antibiotic

significantly *less* than the parent sensitive strain. Sonic extracts also were less reactive with penicillin than similar extracts of the parent culture.

The initial 25-fold increase in resistance, from an $LD_{99.9}$ of 0.024 μg. per ml. to 0.6 μg. per ml., had thus not been associated with a decreased reactivity with penicillin; instead, the resistant strain bound slightly more than the parent culture. The secondary 45-fold increase in resistance (from 0.6 to 28 μg. per ml.) was accompanied by significantly decreased reactivity with penicillin.

3. Diplococcus pneumoniae.—By serial transfer in antibiotic a resistant strain was obtained with an $LD_{99.9}$ of 0.3 μg. per ml., 19 times more resistant than the parent culture. When first tested, on December 3, 1952, that resistant

TABLE IV

Showing That Resistant Variants of Diplococcus pneumoniae May React Normally with Penicillin; and Conversely, That the Combining Affinity with Penicillin May Subsequently Decrease, with No Change in Resistance

	Date of test	$LD_{99.9}$	"Penicillin" bound by bacteria
		μg./ml.	*μg./gm.* dry weigh*
Parent (sensitive) culture	June 1, 1952 to Dec. 16, 1953	0.016	1.44 2.0 1.65
Resistant variants after serial transfer through penicillin	Apr. 16, 1953	0.3	1.35
	" 28, 1953		1.57
	Oct. 6, 1953		0.85
	" 15, 1953		0.62
	Nov. 6, 1953	0.3	0.69
	" 25, 1953		0.465
	Dec. 16, 1953	0.28	0.51

* "Penicillin" bound after exposure for 2 hours to penicillin at 0.01 μg./ml., and retained after washing.

strain bound penicillin to the same degree as the original, sensitive culture (*cf.* Table IV). On continued subculture, despite frequent passages through penicillin, there was no further increase in resistance, the $LD_{99.9}$ remaining at 0.28 to 0.3 μg. per ml. Over this period, there was, however, a significant decrease in the ability of the strain to concentrate the antibiotic from low concentrations (*cf.* Table IV and Fig. 1). In this case, there had been a clear dissociation between the development of resistance and the over-all reactivity of the cell with penicillin. The development of resistance had not originally involved a demonstrably altered reactivity with penicillin; subsequently, there was a progressive decrease in its reactivity with penicillin, with no associated increase in resistance.

4. Streptococcus pyogenes.—When the resistance of the C-203 strain of *Strepto-*

coccus pyogenes was stepped up by serial transfer through antibiotic, the $LD_{99.9}$ concentration finally reached 0.13 μg. per ml., and was not further increased by serial transfer. That resistant strain was significantly less reactive with penicillin at low concentrations than the parent, sensitive culture (Table V); and were it not for the results obtained with *Micrococcus pyogenes* and *Diplococcus pneumoniae*, as described above, one would have been tempted to assume a causal relationship between the decreased reactivity and the enhanced resistance. The sonic extract of the resistant variant also reacted with penicillin

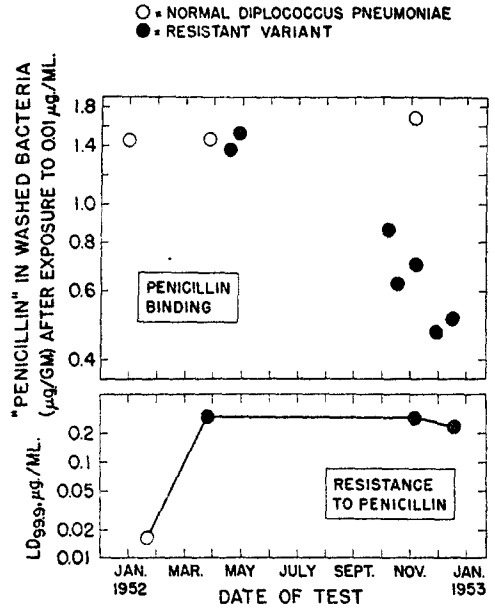

FIG. 1. The development of increased resistance to penicillin in a strain of *Diplococcus pneumoniae* with no accompanying change in its binding affinity for penicillin, and the subsequent decrease in binding affinity with no further increase in resistance.

less strongly than did the normal extract; however, the differences were not as regular or as pronounced as with the intact bacteria.

B. The Rate of Inactivation of Penicillin by Resistant Variants of Normally Sensitive Strains

With the K-12 strain of *Escherichia coli*, it had been found that the free intracellular penicillin was rapidly degraded to a compound which was no longer bactericidal (1). That inactivation could account for the failure of this organism to concentrate the antibiotic, and its penicillin resistance. However, with the other naturally resistant organisms studied, the rate of inactivation of the intracellular penicillin did not exceed that observed with highly sensitive

bacteria. This was found to be true also for the resistant variants under present consideration. As shown in Table VI, there was no significant difference between those resistant variants and the parent sensitive strains with respect to the

TABLE V

The Decreased Affinity for Penicillin of a Resistant Variant of S. pyogenes

Cf. Table I for experimental procedure.

Test materials		$LD_{99.9}$	Concentration of penicillin added to fluid, μg./ml.					
			0.001	0.01	0.1	1	10	100
			"Penicillin bound by bacteria (or by cell-free sonic extract)					
		μg./ml.	μg./gm.	μg./gm.	μg./gm.	μg./gm.	μg./gm.	μg./gm.
Intact organisms	Parent culture	0.006	0.77	3.4	3.71	3	10	25.6
			0.575	3.28	2.44	2.85	7.3	17.1
			0.5	3.0	1.51			
			0.64	2.87	2.55	2.92	8.65	21.4
	Resistant variant	0.13	0.247	1.52	2.8	3.43	6.74	36
			0.19	1.15	1.58	2.59	6.37	29
				1.0	1.41			
				0.9				
				0.85				
				0.61				
			0.22	1.0	1.93	3.0	6.56	32.5
Cell-free sonic extracts	Parent culture	0.006	0.115	1.15	5.57	7.5	9.7	32.4
			0.082	1.03	5.08	7.35	9.4	29.5
			0.082	0.93	4.06	5.34		17
				0.6	2.8	3.45		
			0.093	0.93	5.84	5.91	9.55	26.3
	Resistant variant	0.13	0.074	0.82	4.44	6.77	9.59	52.8
			2.005	0.615	4.41	6.75	9.2	51.8
				0.61	4.17	5.4		
				0.56		4.24		
			0.062	0.65	4.34	5.79	9.4	52.3

rate at which diffusible cellular penicillin was rendered ineffective. Indeed, with one species, *Streptococcus pyogenes*, the highly sensitive parent culture degraded intracellular penicillin more actively than did the resistant variant. The significance of the latter observation is not clear.

Similarly, when sonic extracts of the bacteria were incubated with low con-

TABLE VI

Showing That Penicillin-Resistant Variants of Sensitive Bacteria Degrade Intracellular Penicillin No More Rapidly Than the Parent Sensitive Strains

Radioactive penicillin G at a concentration of 0.01 μg./ml. was added to approximately 3 liters of bacterial culture in the logarithmic phase of growth. After 2 hours at 37°, during which the culture was kept at approximately pH 7.0, the bacteria were collected by centrifugation, the sediments were resuspended in a small volume of the penicillin-broth, and divided into three aliquots, which were recentrifuged in graduated 15 ml. conical tubes. The supernatant fluids were withdrawn, and two of the packed sediments were incubated at 37° for 1 and 4 hours. The third sediment was immediately resuspended in 9 volumes of fresh broth and kept at room temperature for 1 hour. The bacteria were then removed by centrifugation, and the supernatant fluid was sterilized by filtration through either a glass or Selas filter preliminary to the measurement of its total "penicillin" (*i.e.* S^{35}) content, and its actual antibacterial activity, as measured by bioassay with *Streptococcus pyogenes* as the test organism (8). The incubated sediments were similarly treated.

Species tested		Time for which packed penicillin-treated bacteria were incubated at 37° prior to elution	Total cellular "penicillin"*	"Penicillin" eluted on extn. with 9 vols. broth*	Biologically active penicillin in eluate‡	Per cent inactivation of intracellular penicillin
		hrs.	*μg./ml.*	*μg./ml.*	*μg./ml.*	
Streptococcus pyogenes	Normal§	0	1.79	0.0724	0.04	45
		1	2.03	0.071	0.02	72
		4	1.74	0.119	<0.008	>90
	Resistant variant§	0	1.17	0.082	0.06	27
		1	1.07	0.074	0.05	32
		4	1.04	0.074	0.04	46
Diplococcus pneumoniae	Normal	0	1.26	0.102	—	—
		1	1.32	0.087	0.0525	40
		4	1.19	0.091	0.044	52
	Resistant variant	0	1.47	0.074	0.048	34
		1	1.31	0.064	0.044	31
		4	1.26	0.061	0.041	33
Micrococcus pyogenes	Normal	0	2.43	0.107	0.071	35
		1	2.3	0.094	0.0675	29
		4	2.7	0.11	0.0337	70
	Resistant variant	0	1.7	0.085	0.048	44
		1	1.52	0.086	0.022	75
		4	1.6	0.112	<0.008	>90
Streptococcus faecalis	Normal	0	1.46	0.072	0.033	54
		1	1.32	0.069	0.03	57
		4	1.12	0.069	0.0125	81
	Resistant variant	0	1.28	0.053	0.033	35
		1	1.73	0.053	0.0167	68
		4	1.85	0.063	0.0125	79

* Based on radioactivity; *i.e.*, the penicillin equivalent of the S^{35} concentration.

‡ Based on antibacterial assay with *Streptococcus pyogenes* as the test organism, and assuming the extract to contain only fully active penicillin and a totally inactive degradation product.

§ *Cf.* Tables I to V for $LD_{99.9}$ of penicillin for the parent cultures and the resistant variants.

centrations of penicillin, there was no demonstrable difference between the parent cultures and the resistant variants with respect to the degree of inactivation thereby effected (Table VII). From both experiments, one may conclude

TABLE VII

Showing That Sonic Extracts of Penicillin-Resistant Variants Degrade Penicillin No More Actively Than Similar Extracts of the Parent (Sensitive) Strains

Sonic extracts of the various bacterial cultures, diluted to a final concentration of 20 mg. solid/ml., were incubated for 1 hour at 37° with radioactive penicillin at 0.1 μg./ml. The solutions were then centrifuged in the No. 40 head of the Spinco ultracentrifuge for 6 hours at 40,000 R.P.M. (68,000 to 144,000 *g*). The supernatant fluid was sterilized by filtration through a Selas filter, preliminary to the determination of the total "penicillin" (*i.e.* S^{35}) concentration as measured by radioactivity, and actual antibacterial activity, as measured by bioassay with *Streptococcus pyogenes* as the test organism (8). One of two experiments with qualitatively similar results is summarized in the table.

Species tested		"Penicillin" remaining free in solution after incubation of sonic extract with penicillin at 0.1 μg./ml. for 1 hr. at 37°		
		Total*	Biologically active‡	Inactivation
		μg./ml.	*μg./ml.*	*per cent*
Streptococcus pyogenes	Normal	0.066	0.02	70
	Resistant variant	0.062	0.0115	65
Diplococcus pneumoniae	Normal	0.063	0.046	27
	Resistant variant	0.074	0.061	18
Micrococcus pyogenes	Normal	0.0756	0.055	27
	Resistant variant	0.09	0.061	32
Streptococcus faecalis	Normal	0.085	0.05	41
	Resistant variant	0.086	0.033	62

* Based on radioactivity.

‡ Based on bactericidal activity *vs. Streptococcus pyogenes* in dilution assay (8).

§ On the assumption that the supernatant fluid contained only fully active penicillin and a completely inactive degradation product.

that, at least with the four species here studied, the development of increased resistance after selective transfer through increasing concentrations of penicillin was not referable to an enhanced capacity to degrade the antibiotic.

DISCUSSION

It is clear from the present experiments that, with the four bacterial species here studied, when an originally sensitive strain was made resistant by selective

propagation through increasing concentrations of the antibiotic, enhanced resistance was not necessarily related to a decreased reactivity with the antibiotic. This is shown semidiagrammatically in Fig. 2. In some instances, the resistant variant bound more penicillin than normally; in some, its reactivity with penicillin was unchanged; and with others, reactivity was decreased. With *Diplococcus pneumoniae*, the development of increased resistance preceded a decrease in combining affinity; and with *Micrococcus pyogenes* var. *aureus*, as the resistance was progressively increased by selective transfer in antibiotic, the combining affinity with penicillin at first increased and then decreased.

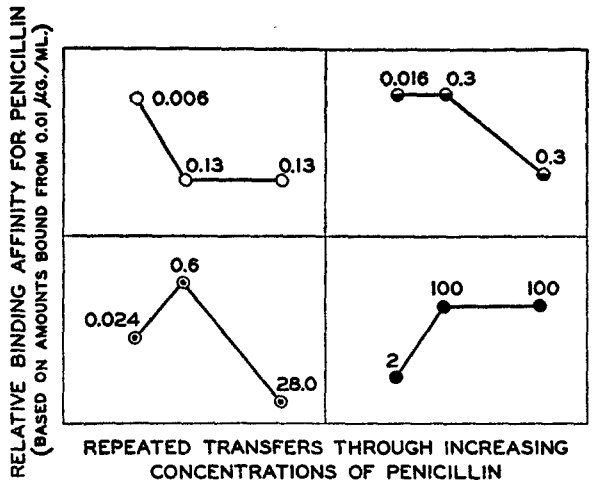

FIG. 2. The absence of a regular correlation between the development of increased resistance to penicillin and the binding affinity for the antibiotic (semidiagrammatic).

The numbers on the curves show $LD_{99.9}$ levels of penicillin. In each curve, the point on the left is the initial stock culture, and the other two points describe resistant variants produced by serial transfer through increasing concentrations of antibiotic. ○, *Streptococcus pyogenes*; ◉, *Micrococcus pyogenes*; ◒, *Diplococcus pneumoniae*; ●, *Streptococcus faecalis*.

Similarly, the development of increased resistance was not accompanied by an increased capacity to degrade the antibiotic, either extracellularly or within the cell.

One may conclude that, although the penicillin sensitivity of bacterial strains and species as they occur in nature is usually related to the over-all combining affinity of the cells with the antibiotic, and occasionally may be referable to its intracellular degradation, other mechanisms are often responsible for the increased resistance produced in originally sensitive strains by selective propagation in the presence of penicillin. Indeed, it is entirely possible that multiple mechanisms are operative, varying from species to species, and perhaps from strain to strain.

(*a*) Thus, it is possible that the resistant cell no longer requires the metabolic activity which is normally inhibited by penicillin at the usual bactericidal concentration. Higher concentrations of antibiotic would kill the resistant cell by inactivating other vital components, sensitive only to those higher concentrations. Under these circumstances, a progressive increase in the penicillin resistance of a given strain would reflect a progressive qualitative change in the metabolic function inhibited by penicillin and responsible for the death of the cell.

(*b*) The metabolic activity affected by penicillin may be vital to both the normal cell and its resistant variant, and inhibited to the same degree by penicillin in both; but the resistant cell may have a quantitatively smaller requirement for the product of the reaction affected. Thus, the normal sensitive cell might die if the particular metabolic function were decreased to *e.g.* 50 per cent at a penicillin concentration of 0.01 μg. per ml.; while the resistant cell might survive even a 90 per cent inhibition by a concentration of *e.g.* 0.1 μg. per ml.

(*c*) In those instances in which the resistant variant is actually more reactive with penicillin than the parent cell (*cf.*, *Streptococcus faecalis; Micrococcus pyogenes*, strain B) the resistant cell may contain (or produce) a larger amount of the vulnerable component affected by penicillin, necessitating a larger concentration of antibiotic in order to effect the necessary degree of inhibition. One must, however, then assume that the specific component accounts for a major part of the penicillin bound by the cell.

(*d*) In the few instances in which the development of increased resistance to penicillin was accompanied by a significant decrease in combining affinity for the antibiotic (*cf. Streptococcus pyogenes; Mycrococcus pyogenes* var. D), there may have been a causal relationship; *i.e.*, the cell may have become resistant because the vulnerable components were less reactive with penicillin. Higher concentrations would then be necessary in order to effect their inactivation.

(*e*) As an extension of the foregoing, the possibility remains that the development of increased resistance is regularly related to the decreased penicillin reactivity of the specific vulnerable component(s) which is inactivated by the antibiotic and which is vital to the cell; and the apparently contrary evidence of the present paper can be reconciled with that thesis. The binding of penicillin by bacteria, or by bacterial extracts, may involve a number of different cellular components, and may reflect only in part the reactivity of the specific vital component(s) inactivated by penicillin. Of the 1500 to 3000 molecules of penicillin which are bound by each bacterial cell at the $LD_{99.9}$ concentration, only *e.g.* 100 may be in combination with the component(s) specifically inactivated by the antibiotic, and responsible for the death of the cell. The resistant organism may differ from the sensitive, parent cell in that the vulnerable cell constituent(s) has undergone modification, and is no longer as reactive with the penicillin. This could be masked by the unchanged binding affinity of the other

penicillin-reactive cellular components, and might not cause a demonstrable decrease in the over-all binding affinity of the cell; nevertheless, higher concentrations would be necessary in order to bring about the inactivation of the specific factor, and the death of the cell.

It is of interest in this connection that trypanosomes which have become resistant to certain arsenicals no longer bind the specific compounds to which they are resistant (*cf.* references 5 and 6); and the same finding has been reported in the case of mycobacteria which have become resistant to isoniazid (7). With the arsenicals, the failure of the resistant organisms to bind the drug has been tentatively explained on the basis of specific permeability changes With the penicillin-resistant variant here studied, however, there was no indication that increased resistance was related to decreased permeability. It would be of interest to study the reactivity of cell-free extracts of those drug-resistant trypanosomes and mycobacteria in order to determine whether the primary determinant of their enhanced resistance is in fact an altered permeability, or whether, as with naturally penicillin-resistant bacteria, the vulnerable cellular components have become less reactive with the chemotherapeutic agent.

Indeed, the decreased reactivity of a vulnerable cell component (*e.g.* an enzyme protein) with a drug may be a fairly common basis for resistance to its cytotoxic action. Thus, Davis and Maas (9) studied mutant strains of *Escherichia coli* resistant to *p*-nitrobenzoic acid, to sulfanilamide, and to a number of sulfanilamide derivatives. In a cogent analysis of the cross-resistance relationships in those mutants, of the competitive ratios of drug:metabolite, and of the PAB requirement of the resistant strains, the only explanation of resistance uniformly consistent with the experimental data was that an enzyme protein of the resistant cells had a decreased affinity for the inhibitor as compared with the normal substrate.

SUMMARY

1. In a previous study, the differing sensitivity of bacterial strains as they occur in nature appeared to be correlated with their correspondingly differing reactivity with penicillin. Presumably, the over-all reactivity of the cell with penicillin paralleled that of the vulnerable cell component(s). However, when penicillin-resistant variants of these strains (*Streptococcus pyogenes, Micrococcus pyogenes, Diplococcus pneumoniae,* and *Streptococcus faecalis*) were produced by serial passage through increasing concentrations of antibiotic, this correlation between resistance and the ability of the cell to bind penicillin was no longer apparent. Some resistant variants bound more penicillin than the parent, sensitive cell (*Streptococcus faecalis, Micrococcus pyogenes*); some were unchanged in their reactivity (*Diplococcus pneumoniae, Micrococcus pyogenes*); and some bound less (*Streptococcus pyogenes, Micrococcus pyogenes*). One re-

sistant variant of *Micrococcus pyogenes* at first showed enhanced reactivity with penicillin; on continued passage through antibiotic, there was a further increase in resistance, but now associated with a significantly decreased reactivity. In the case of *Diplococcus pneumoniae*, a resistant variant at first reacted normally with penicillin; on continued passage in antibiotic, its binding affinity for penicillin gradually decreased, but with no associated further increase in resistance.

2. The reactivity with penicillin of cell-free sonic extracts of the resistant variants paralleled that of the intact organisms. Permeability considerations therefore did not seem involved in the increased resistance produced by serial passage in antibiotic.

3. The penicillin-resistant variants did not have an enhanced capacity to degrade the free intracellular antibiotic.

4. Possible alternative explanations are discussed in the text.

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