PROPERTIES OF THE PENICILLIN BINDING COMPONENT OF MICROCOCCUS PYOGENES[1]

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The antibiotic effect of penicillin on bacterial cells is known to involve the inhibition of a process necessary for cell division but not primarily connected with respiration (Florey *et al.*, 1949). Previous work in which the primary bacteriostatic action of penicillin has been apparently identified with a disturbance in amino acid (Gale, 1952) or nucleotide metabolism (Gros and Macheboeuf, 1952; Mitchell and Moyle, 1951) has been performed under conditions which have not allowed primary and secondary effects to be clearly distinguished. Maass and Johnson (1949*a*, *b*) and Rowley *et al.* (1950) have demonstrated that washed cells of *Micrococcus pyogenes* var *aureus*, strain H, specifically and irreversibly bind small but relatively constant amounts [approximately 0.8 *u* per g of wet cells (Maass and Johnson, 1949*a*)] of penicillin (S^{35} labeled), and that this uptake is associated with an early and possibly the primary step in growth inhibition. A sensitive criterion of inhibition is thus available.

The mechanism of action of penicillin has been approached in this investigation through the use of this criterion by studying (a) the binding of penicillin by *M. pyogenes* var *aureus* cell-free extracts, and (b) the effect of penicillin derivatives and various metabolic inhibitors on the whole cell uptake of S^{35} penicillin by *M. pyogenes*.

METHODS

The culture employed was *M. pyogenes* var *aureus*, strain H. Cells were produced by growth for 6 hours on the medium of Maass and Johnson (1949*a*) in shake flask culture. Such cells were approximately 25 per cent dry weight and according to Maass and Johnson (1949*a*) have a population density of 1.1×10^{12} cells per g (wet weight).

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Cell binding of penicillin was accomplished by incubating washed cells for 30 minutes with one to two units of penicillin for each ml of a cell suspension containing 0.1 to 0.5 g of wet cells. Excess penicillin was removed by suspending the cells in one per cent KH_2PO_4 adjusted to pH 6.3, centrifuging, and repeating this procedure twice. Although *M. pyogenes* cells usually bind an average of 0.8 *u* per g, occasional batches of cells have bound as much as 1.1 *u* per g.

A *M. pyogenes* cup-plate assay sensitive to 0.06 to 0.07 *u* per ml was used to determine penicillin. S^{35} labeled penicillin was determined either by bioassay or by counting (see below). The unlabeled penicillin used was the crystalline potassium salt of benzyl penicillin. Two preparations of S^{35} labeled penicillin were used. In the first experiments, a relatively impure product produced biosynthetically by a previously described method (Maass and Johnson, 1949*a*) was used. Later the crystalline potassium (originally 135 mc per g) and N-piperidine (originally 146 mc per g) salts of benzyl penicillin were obtained from Abbott Laboratories. The latter preparations were 90 to 100 per cent pure by bioassay, and the potassium salt was 97 per cent benzyl penicillin as determined by paper chromatography.

Radioactivity (S^{35}) was determined by counting samples air dried on 5 sq cm copper planchets. Counts were corrected for coincidence and self-absorption losses and for background.

Derivatives of benzyl penicillin were prepared from the crystalline potassium salt of benzyl penicillin by the established methods cited below (Clark *et al.*, 1949).

Penicilloic acid was prepared (Clark *et al.*, 1949) by the hydrolysis of penicillin at pH 11 until no biological activity remained. The product was not isolated. Penilloic acid was prepared (Clark *et al.*, 1949) by the α-decarboxylation of penicilloic acid at pH 2.5. Penillic acid was prepared (Clark *et al.*, 1949) by the pH 2.5-catalyzed rearrangement of penicillin. The crystalline derivative was isolated. Penillonic acid was pre-

pared (Clark *et al.*, 1949) by isomerization of penicillin by overnight refluxing of penicillin methyl ester in toluene. The crystalline penillonic methyl ester was isolated and saponified before use. Penicillamine was derived (Clark *et al.*, 1949) by the aqueous $HgCl_2$ degradation of penicilloic acid, regeneration of the resulting mercuric complex with H_2S, and isolation as the crystalline hydrochloride salt. Desthiopenicillin was prepared (Clark *et al.*, 1949) by the reductive desulfuration of penicillin with Raney nickel. The initial product was reprecipitated from an ethanol-water mixture before use. Cysteine penicillin was prepared (Clark *et al.*, 1949) by the aqueous inactivation of penicillin with excess cysteine and isolated as the crystalline dibenzylamine salt.

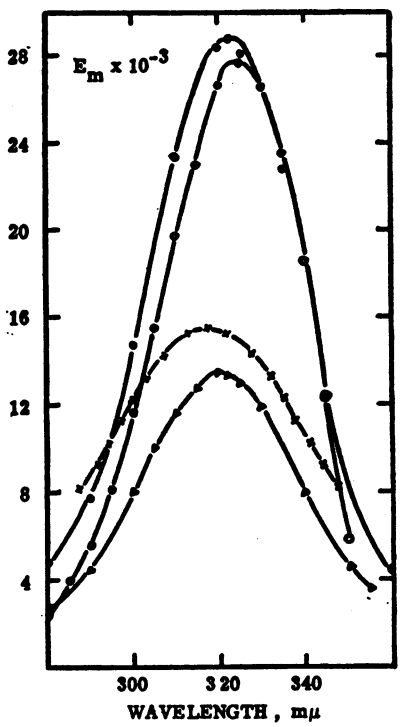


Figure 1. The spectra of synthetic and pH 4.6 acetate prepared penicillenic acid and of penicillenic acid-HgCl complexes.

●—●—● = pH 4.6 acetate prepared penicillenic acid in aqueous pH 6.3 acetate buffer. E_m multiplied by two for purposes of comparison.

O—O—O = synthetic S-benzyl, benzyl penicillenic acid in methanol (Clark *et al.*, 1949).

X—X—X = benzyl penicillenic-HgCl complex in ethyl acetate; calculated for a synthetic preparation of E_m = 15,500 at 320 mμ (Clark *et al.*, 1949).

▲—▲—▲ = product formed by reaction of $HgCl_2$ with benzyl penicillin, and dissolved in dioxane.

Penicillenic acid was prepared by two methods. The method of Herriot (1946) was found to give a high and consistent yield only after the addition of Cu^{++}. Penicillenic acid was formed in 65 to 70 per cent yield when a solution of 2.32 mg per ml of potassium benzyl penicillin in pH 4.6, 0.44 M acetate buffer (potassium), and 3.3 × 10^{-5} M $CuSO_4$ was heated in a glass capped tube for 15 minutes in a boiling water bath.

Penicillenic acid was estimated by light absorption at 321 mμ from an E_m of 21,300 (Clark *et al.*, 1949). Penicillenic acid was also prepared (Clark *et al.*, 1949) by the aqueous $HgCl_2$ degradation of penicillin. The penicillenic HgCl complex was isolated and decomposed in pH 6.8, 0.3 M phosphate buffer with H_2S. The mercuric complex exhibited 84 per cent of the described

TABLE 1

Release of penicillin binding component and of bound S^{35} penicillin from whole cells by alumina grinding

EXPERIMENT	RELEASE*	FREE PENICILLIN†	RELEASE
	u per g	*u per ml*	*per cent maximum*
1	0.71	0.45	86
2	0.47	0.24	57
3	0.70	0.83	88
4	0.45	0.13	50
5	0.48	0.16	60
6	0.46	—	68
7	0.38	—	44
8	0.36	—	53
9	0.71	—	79

* In experiments 1–5, release of the penicillin binding component from normal cells was determined by addition of penicillin to the extract and bioassay. In experiments 6–9, release of bound S^{35} penicillin from penicillin treated cells was determined by counting the extract obtained by centrifuging at 3,600 G for 15 minutes.

† Penicillin remaining unbound in extracts.

Maximum release is that expected on the basis of whole cell binding. Release for experiments 1–5 has been calculated from the originally determined basis of *u* per ml to *u* per g of ground cells from which the extract was derived.

E_m, but the yield of the H_2S regenerated product was only 9 per cent of that expected from the complex. The spectra of authentic and the above described preparations are found in figure 1.

Cell-free extracts of normal cells were prepared by grinding one g of wet-packed *M. pyogenes* cells with two g of alumina in a cold mortar immersed in an ice bath. The grindings were suspended in 4 volumes (per volume of cells ground) of one per cent KH_2PO_4, adjusted to pH 6.3 to 6.8 with 20 per cent H_3PO_4 and centrifuged at 3,300 G for 20 minutes. The clear or opalescent supernatant was removed, the residue again extracted with buffer, and the supernatants combined. S^{35} penicillin bound by whole cells was released similarly. Cell-bound penicillin is apparently a complex of penicillin with a cell component (Maass and Johnson, 1949*b*). It has been found in this study that whole, washed *M. pyogenes* cells are permeable to but do not bind cell bound S^{35} penicillin prepared in soluble form as just described.

To determine the penicillin binding capacity (the amount of binding component) of cell extracts, penicillin was added to 1.0 to 2.0 ml of pH 6.3 extract, the mixture incubated as desired, and then heated in a steam bath for 30 seconds to raise the temperature to 95 C, and maintained at 95 to 100 C for 60 seconds. Samples were then rapidly cooled and submitted for assay of the free penicillin. Controls containing only penicillin in pH 6.3 buffer showed no destruction of activity by heating in this manner.

TABLE 2

Effect of incubation time and penicillin concentration on penicillin uptake of cell-free extracts

INCUBATION TIME	FINAL PENICILLIN CONCENTRATION	PENICILLIN UPTAKE	PER CENT OF WHOLE CELL UPTAKE
min	*u per ml*	*u per g*	
3	0.25	0.58	71
20	0.27	0.47	57
180	0.24	0.64	78
5	0.13	0.70	85
5	0.32	0.71	87
5	0.86	0.70	85

Whole cell uptake was 0.82 *u* per g of wet cells, the cell extract being equivalent to 0.17 g cells per ml. The extract incubated for 5 minutes was prepared separately.

The appropriate volume of concentrated penicillin solution was added to the cell extract with a micropipette, 1.0 ml samples removed after the indicated incubation, immediately heated as described in the text, cooled, and submitted for assay.

RESULTS

Binding of penicillin by M. pyogenes cell-free extracts. M. pyogenes extracts when tested for penicillin binding capacity were found to bind 50 to 88 per cent, and in no instance more than 100 per cent, of the penicillin that would have been bound by the whole cells from which they were derived. Typical results are shown in table 1. Results for the release of cell bound S^{35} penicillin also are included. As much as 22 per cent of the bound S^{35} penicillin has been recovered from cell debris in some experiments. Whole cell uptake of penicillin is practically instantaneous at penicillin concentrations of several tenths unit per ml or greater, and the amount of uptake is independent of the concentration employed (Maass and Johnson, 1949*a*). The data in table 2 show that the total extract uptake occurs within the time necessary to remove the initial sample and that the amount of binding is unaffected by the penicillin concentration up to 0.86 *u* per ml. Thus, the same agent appears to be responsible for both cell extract and whole cell binding. Further evidence for this is shown in table 3. When whole cells are treated with penicillin, washed thoroughly to remove excess penicillin, and ground, extracts of such preparations bind little or no penicillin even after a two hour incubation. The penicillin binding component is heat-labile, approximately 80 per cent of the activity being

TABLE 3

Penicillin binding by extract of penicillin treated, alumina-ground cells

TIME	OBSERVED BINDING	
min	*u per ml*	*u per g*
0	−0.01	−0.07
5	0.01	0.07
60	−0.02	−0.14
120	0.00	0.00

Penicillin treated whole cells bound 0.70 *u* per g of wet cells. A corresponding extract uptake would be 0.14 *u* per ml assuming extract binding to be as great as that of whole cells. Three-tenths *u* of penicillin was added per 1.0 ml of cell extract.

TABLE 4

Solubility of bound S^{35} penicillin in organic solvents

SOLVENT	EXTRACTION pH	BOUND S^{35} PENICILLIN EXTRACTED BY SOLVENT
		per cent of total
Ethyl ether	2.0	18
n-Butanol	3.0	10
	8.5	5
Ethyl acetate	3.0	7
	8.5	1

Bound penicillin was prepared by pH 6.3 buffer extraction of alumina-ground, penicillin treated cells. The extract (5.0 ml) was adjusted to the indicated pH, shaken with an equal volume of solvent, the solvent separated, evaporated in the presence of pH 6.3 phosphate buffer, and aliquots of the buffer removed for counting.

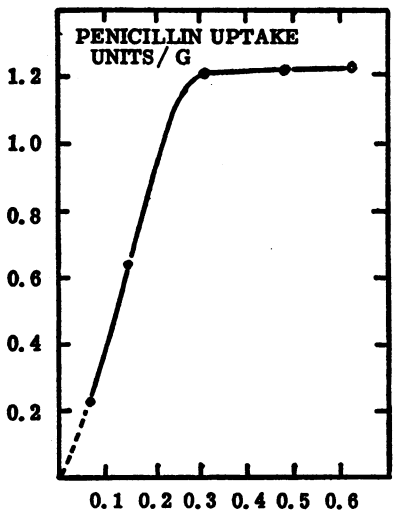


Figure 2. The effect of penicillin concentration on the uptake of penicillin by *Saccharomyces carlsbergensis* cell-free extract.

Cell extract was prepared by the method used for *Micrococcus pyogenes* cell-free extracts. The extracts after addition of penicillin with a micropipette were incubated at room temperature for one hour, heated in a steam bath (see text), cooled rapidly, and submitted for assay.

destroyed (at pH 6.3) by the heat treatment described under Methods. The binding component in extracts is at least 90 per cent soluble in 3 per cent trichloroacetic acid (TCA) when addition of trichloroacetic acid is followed by rapid centrifugation and readjustment to pH 6.3. Treatment with crystalline trypsin and chymotrypsin in the presence of toluene at pH 8.2 for 24 hours resulted in only a 10 per cent loss of activity. The component remains 70 per cent soluble after pH 2.5 precipitation (H_3PO_4), but cell-bound S^{35} penicillin extracted from ground cells was 70 per cent precipitable by such treatment.

The organic solvent solubility of cell-bound S^{35} penicillin extracted from ground cells is indicated in table 4. In all cases the solubility is low. Ninety-three to 100 per cent recovery of S^{35} was obtained in all instances. Acetone-ether treatment of cells at ice bath temperatures also did not remove the component. These results are in disagreement with the recent findings of Cooper (1953) who has reported the binding component and cell-bound penicillin in his preparations to be lipid soluble. Difference in methods of preparation may account for this discrepancy.

Cell-free extracts of fresh yeast cells show a different type of penicillin uptake. The effect of concentration on this penicillin binding is shown in figure 2. Although the amount of uptake is

TABLE 5

Effect of penicillin derivatives on the whole cell uptake of S^{35} penicillin

(See figure 3 for derivative structure)

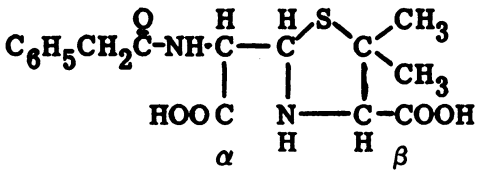
DERIVATIVE	DERIVATIVE CONCENTRATION	S^{35} PENICILLIN UPTAKE	PER CENT OF CONTROL*
	μg per ml	*u per g*	
1. Penicilloic acid	10	1.18	94
2. Penilloic acid	9	1.19	94
3. Penillic acid	12	1.20	95
4. Desthiopenicillin	20	0.69	96
5. Cysteine penicillin	21	0.77	104
6. Penicillamine	12	0.13	90
7. Penicillenic acid	12	0.20	40†
8. Penillonic acid	12	0.77	104

* Control uptake (no treatment), *u* per g: for no. 1, 2, 3, 6 = 1.26; for no. 4, 5, 7, 8 = 0.74.

† Corrected for non-S^{35} penicillin present in the preparation.

Cells suspended in pH 6.3 phosphate buffer containing the indicated concentration of derivative (in pH 6.3 buffer) were incubated one hour, washed in buffer, treated with S^{35} penicillin for 30 minutes, washed, suspended in buffer, and counted.

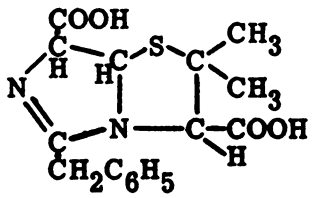
All cells (0.09 to 0.15 g per ml) were treated with 0.7 to 0.8 *u* per ml of penicillin. In no case was more than 0.2 *u* per ml bound by the cells present.



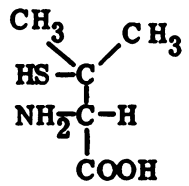
Penilloic Acid = α-decarboxylated penicilloic acid

Cysteine Penicillin = α-HSCH$_2$CH(NH-)COOH derivative of penicilloic acid

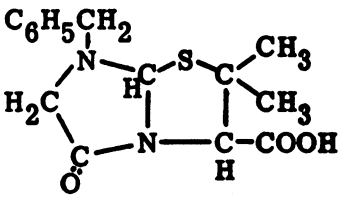
Penicilloic Acid



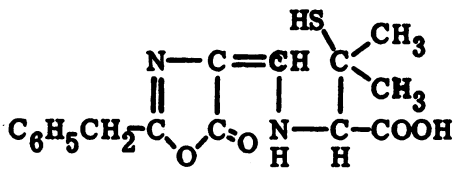
Penillic Acid



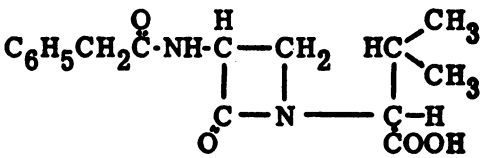
Penicillamine



Penillonic Acid



Penicillenic Acid



Desthiopenicillin

Figure 3. Structures of the benzyl penicillin derivatives tested for inhibition of penicillin uptake by whole cells.

similar to that of *M. pyogenes* extracts, concentration exerts a pronounced effect; in addition, the uptake is not substantially heat-labile. Maass and Johnson (1949*a*) had shown previously that the penicillin resistant *Saccharomyces cerevisiae* did not bind S^{35} penicillin and was not penicillin permeable. *Saccharomyces carlsbergensis* also has been found to bind only 0.01 *u* or less per g of wet cells.

Recent experiments in this laboratory (Schepartz and Johnson, 1953) indicate that *M. pyogenes* cell extracts also inhibit a type of penicillin binding that is concentration dependent but which is not reversible. No satisfactory explanation for this behavior has been found. Few *et al.* (1952, 1953) have reported the use of an electrodialysis method to determine specific penicillin binding by cell-free preparations and that under their conditions of cell rupture most of the specific binding (affected only slightly by penicillin concentration) resides in the cell wall fraction.

Effect of penicillin derivatives on the S^{35} penicillin uptake of whole cells. Structural derivatives of penicillin which have no biological activity

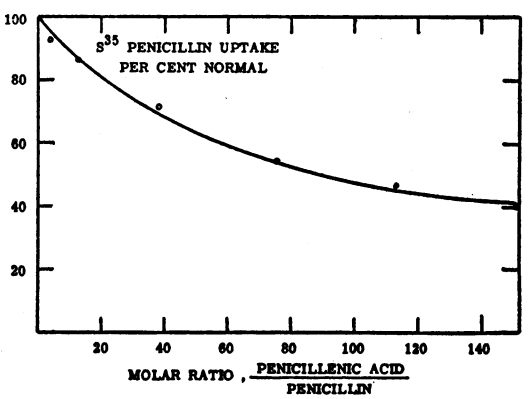


Figure 4. The inhibition of S^{35} penicillin cell uptake by penicillenic acid.

Cells (0.27 g per 3.2 ml) were incubated for one hour in pH 6.3 phosphate buffer containing the indicated mixtures of penicillenic acid and S^{35} penicillin, washed, and resuspended in buffer for counting. The S^{35} penicillin concentration varied from 0.11 to 1.6 μg per 3.2 ml, an excess of penicillin always being present. The penicillenic acid concentration varied from 5.5 to 50 μg per 3.2 ml.

should not interfere with penicillin binding by *M. pyogenes* cells if this binding is a direct cause of growth inhibition. Eight derivatives were tested for their ability to interfere with the normal cell binding of S^{35} penicillin. Washed cells were treated with the derivative, washed, and then treated with S^{35} penicillin since the ability to block the cell site necessary for binding penicillin was the critical property to be tested. The concentration of derivative applied was 300 to 600 times the minimum molar penicillin concentration necessary for inhibition. Concentrated solutions (one to two mg per ml) of all derivatives were found to be biologically inactive by plate assay. The results are summarized in table 5. Penicillamine produced a small effect. Only penicillenic acid exhibited a substantial inhibition effect. This effect was studied in more detail.

Since actual cell uptake of penicillenic acid appeared to be involved, S^{35} penicillenic acid (acetate-Cu^{++} method, from S^{35} penicillin) was employed. An actual uptake of 1.0 μg per g of wet cells was detected after cells were treated with 4.9 μg per ml of S^{35} penicillenic acid and washed. A 50 per cent inhibition of S^{35} penicillin uptake occurred. This inhibitive effect can be demonstrated also when penicillenic acid and S^{35} penicillin are present together, as in figure 4. Acetate alone (present in the penicillenic acid preparation) at pH 5.0 to 7.5 did not affect whole cell penicillin uptake. The cell uptake of S^{35} penicillenic acid was found to be dependent on the concentration applied. In the presence of 0.53 μg per ml, an uptake of 0.12 μg per g was observed, but at 10.2 μg per ml, a 2.8 μg per g uptake occurred; therefore, more penicillenic acid was bound than penicillin.

Cells treated with unlabeled penicillin, washed, and treated with S^{35} penicillenic acid bound 2.63 μg per g whereas normal cells bound 2.83 μg per g. If the two molecules have the same binding site, only 2.28 μg per g uptake should have occurred; therefore, penicillenic acid is apparently bound at a site different from that at which penicillin is finally bound. If an equilibrium reaction is involved in penicillenic acid uptake, the equilibrium is reached slowly since incubation of the unlabeled acid (8.1 μg per ml) with cell-bound S^{35} penicillenic acid (1.96 μg per g wet cells; 0.08 g cells per ml) for one hour resulted in only 9 per cent of the calculated exchange (1.34 μg per g instead of 0.12 μg per g). Penicillenic acid will not displace bound S^{35} penicillin from whole cells.

Since the acetate penicillenic acid preparation was not pure, penicillenic acid prepared by the previously mentioned $HgCl_2$ method was tested but found to be only weakly inhibitory. Penicillin uptake was inhibited only 18 per cent by 36 μg per ml. However, the inhibiting power of the acetate preparation is diminished considerably by pH 11 or benzylamine treatment (reagents which decompose penicillenic acid). A closely related derivative or a different *cis-trans*-isomer may be involved.

A number of metabolic inhibitors also have been tested for their effect on the S^{35} penicillin uptake of washed *M. pyogenes* cells. Arsenate, fluoride, borate, hydrogen sulfide, iodoacetate, and arsenite have no significant effect on uptake by washed cells at the 0.1 M level for one hour (H_2S passed through cell suspension for 15 minutes). The following compounds caused the denoted percentage inhibition of uptake: 0.1 N 2,3-dimercaptopropanol (BAL), 47; 0.1 M cyanide, 27; 0.1 M thioglycolate, 12; 0.1 M cysteine, 12. All of the inhibiting compounds possess both reducing and metal complexing properties. Dimercaptopropanol inhibition is not lessened appreciably by the removal of dimercaptopropanol before the addition of penicillin. The dimercaptopropanol inhibition can be reversed by several metal ions and by aeration as is shown in table 6. Previous workers (Pratt and Dufrenoy, 1948; Pital *et al.*, 1953) have indicated that cells growing in the presence of Co^{++} are much more sensitive to penicillin than in its absence. In view of the relatively high metal ion concentration used, the restorative effect indicated in table 6 may occur by a mechanism not normally operative in the cell. It is also of interest that incubation of S^{35} penicillin treated and washed *M. pyogenes* cells with 0.1 M dimercaptopropanol for an hour causes a solubilization of 22 per cent of the bound S^{35}.

A reversibly reducible cell group (but one normally oxidized in washed cells) appears to be involved in the penicillin binding reaction. The inhibition of penicillin uptake produced by the penicillenic acid preparation also supports this interpretation and suggests that this inhibitor interferes with penicillin uptake by blocking a cell site different from that finally occupied by penicillin.

TABLE 6

Release of the dimercaptopropanol (BAL) inhibition of S^{35} penicillin uptake by aeration and metal ions

TREATMENT	S^{35} PENICILLIN UPTAKE	PER CENT OF CONTROL UPTAKE
	u per g	
Control (phosphate buffer only)	1.21	(100)
BAL extraction*	0.64	53
BAL extraction†	0.72	60
Metal addition: (to cells, BAL treated and washed)		
Fe^{+++}	0.72	60
Cu^{++}	0.99	82
Mn^{++}	1.17	97
Co^{++}	1.92	159
Mg^{++}	0.72	60
Zn^{++}	0.56	46
Aeration (30 minutes) of BAL treated, unwashed cells	0.94	78
Aeration (40 minutes) of BAL treated, washed cells	1.07‡	98
Control + Co^{++}	1.19	99
Control + Mn^{++}	1.16	98

* Cells unwashed before addition of penicillin.

† Cells washed with pH 6.3 phosphate buffer before addition of penicillin.

‡ Control uptake = 1.09 *u* per g.

Cells were incubated for 4 hours with freshly prepared approximately 0.1 M dimercaptopropanol (extracted from a glass sealed arachis oil dimercaptopropanol solution) in pH 6.3 phosphate buffer, the cells (0.065 g wet cells per ml) collected by centrifugation, and the treatment repeated for a one hour and then a half hour interval. Excess penicillin was added (before or after washing, and after metal treatment), the cells incubated 30 minutes, penicillinase added to prevent possible binding during preparation for counting, and the unbound S^{35} removed by washing.

Washed dimercaptopropanol treated cells were incubated 15 minutes with the solutions of metallic ions (75 μg M^+ and 0.065 g cells per ml) washed before addition of penicillin. Filtered, compressed air was used for aeration. Double-distilled water was used for the preparation of metallic salts solutions and for washing dimercaptopropanol treated cells.

SUMMARY

Cell-free extracts of alumina-ground *Micrococcus pyogenes* var *aureus*, strain H, cells bind penicillin. This binding, similar in magnitude to that of whole cells, is independent of the penicillin concentration and appears to result from reaction with the same agent responsible for whole cell binding. Extracts of penicillin treated cells do not bind penicillin.

Treatment of whole cells with a number of penicillin derivatives does not prevent whole cell binding of penicillin, but a preparation of penicillenic acid does cause appreciable inhibition of binding. An actual uptake of S^{35} may be demonstrated when cells are exposed to an S^{35} penicillenic acid preparation.

Dimercaptopropanol and cyanide cause appreciable inhibition of whole cell penicillin binding. The dimercaptopropanol inhibition may be relieved completely by aeration or treatment of the cells with certain metal ions.

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