PENICILLIN UPTAKE BY BACTERIAL CELLS[1]

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In the earlier literature (Hobby, Meyer, and Chaffee, 1942) it has been recorded that penicillin acted on bacterial cells without being absorbed or adsorbed by them. In these studies the bacterial concentrations used were so small that a penicillin uptake of a few thousand molecules per cell would not have been detected. Recently, Pasynskiĭ and Kastorskaya (1947) have reported that a culture of *Staphylococcus aureus* concentrated 500 times and incubated for 18 hours at 37 C with 0.8 to 1.0 unit per ml of penicillin adsorbed an amount of penicillin calculated as 1.5×10^{-6} times the wet weight of the bacteria. This would be equivalent to 1,000 molecules of penicillin per bacterial cell. On the other hand, Rowley *et al.* (1948), who used radioactive penicillin in order to be able to detect small amounts of penicillin, reported that less than 10 molecules of penicillin were taken up per cell by *S. aureus*. In their experiments the cells were washed after being equilibrated with penicillin and could have lost reversibly absorbed penicillin. The purpose of the experiments reported here was to determine the manner in which penicillin is taken up by penicillin-sensitive *S. aureus* cells.

If to a bacterial cell paste a penicillin solution is added, the volume of which is equal to the volume of the total water in the cells, the proportion of the original penicillin remaining in the supernatant solution will depend on the behavior of the cells toward penicillin. If no penicillin is destroyed or absorbed by the cell, the extracellular penicillin concentration will remain unchanged. If adsorption takes place, any proportion of the penicillin may be adsorbed, but at low penicillin concentrations the amount adsorbed should be proportional to the concentration, whereas at sufficiently high concentrations saturation of the adsorbing surfaces should occur. If the penicillin is not adsorbed, but penetrates the cell wall and distributes itself between the intracellular and extracellular water, the penicillin concentration in the supernatant will fall to half its original value regardless of the penicillin concentration. If penicillin inactivation by a stoichiometric reaction takes place, the amount of penicillin inactivated should be constant and independent of time or penicillin concentration. If catalytic inactivation occurs, the amount of penicillin destroyed should increase with time.

The first series of experiments conducted were limited to cell suspensions and penicillin solutions of concentrations such that the detection of penicillin by

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biological assay methods was possible. In the second series of experiments penicillin in which the sulfur was replaced by radioactive S^{35} of high specific activity was employed, which made possible the detection of very small concentrations of penicillin both in the cells and in the liquid surrounding them.

METHODS

Experiments involving nonradioactive penicillin. The penicillin in all experiments was pure penicillin G sodium salt dissolved in pH 6.1 phosphate buffer. Commercial bakers' yeast was used as a source of cells for experiments with *Saccharomyces cerevisiae.*

Cells for experiments with *Staphylococcus aureus* (strains 209P and H) were grown in aerated culture in 5-gallon bottles on a glucose, peptone, yeast extract, beef extract medium. Ten liters of medium were inoculated with 100 ml of a 24-hour broth culture. Growth was allowed to proceed for 16 to 18 hours before the cells were harvested in a Sharples centrifuge. Cells thus harvested averaged 81 per cent water and had a specific gravity of 1.12. The cells were weighed, and a volume of phosphate buffer was added equal to the volume of the cells as determined from their weight and specific gravity. Penicillin in varying concentrations was added to 10-ml samples of this suspension in test tubes and mixed with the cells; after incubation the tubes were centrifuged and the supernatant was removed and analyzed.

The standard cup-plate assay was used for the analysis of the supernatant solutions. *S. aureus* H was the test organism for most of the analyses involving penicillin concentrations greater than 1 unit per ml. An assay (Burke, 1947) employing *Sarcina lutea* as the test organism was used for analyses from 0.025 units per ml, the lowest limit of sensitivity, to approximately 1 unit per ml. The results obtained with the two assays checked closely.

Experiments involving radioactive penicillin. The fermentation for the production of radioactive penicillin was conducted according to the method of Singh and Johnson (1948). Approximately 8 millicuries of carrier-free S^{35} as sulfuric acid were added to the synthetic medium before sterilization. The sulfur content of the medium was reduced to 212 μg per ml to increase the relative specific activity of the S^{35}. Penicillin G precursor, in the form of sodium phenylacetate, was added at 12-hour intervals during the course of the fermentation. Radioactive penicillin was extracted from the fermentation mixture at pH 2 with ether. The ether solution was then extracted with 2 per cent K_2HPO_4. The phosphate extract contained 1,088 units per ml of penicillin, shown by filter paper chromatography to be 90 to 95 per cent penicillin G, which had a relative specific activity of 3,700 observed counts per minute per unit of penicillin. Since the specific activity of the sulfur in the medium was known, it could be calculated that a maximum of 8 per cent of the radioactivity of the final extract was not due to the penicillin present.

Determinatious of the radioactivity of samples from the fermentation were made directly on 0.2-ml portions of solution evaporated to dryness on glass cover slips. Corrections were made in the counts for resolution time of the counter

tube, for background, and for radioactive decay when it was necessary to compare samples counted several days apart.

To compare determinations of radioactive penicillin in cells of *S. aureus* and surrounding solutions, however, it was necessary to reduce all samples to uniformity of composition and size. For this purpose, the samples were oxidized by strong alkaline fusion (Bailey, 1937). Sulfur was precipitated as barium sulfate in centrifuge tubes with bottoms formed by detachable flat celluloid disks (Maass, Larson, and Gordon, 1949). After centrifugation and washing, the precipitates on the disks were dried, weighed, and counted. Correction for counts absorbed by the sample was necessary here, in addition to the corrections mentioned above, since the sample was no longer infinitely small.

RESULTS

The results obtained when *S. cerevisiae* cells are incubated at pH 6.1 with equal volumes of buffer containing varying concentrations of penicillin are shown in figure 1. No significant change in the penicillin concentration in the buffer after equilibration may be observed, and therefore no adsorption of penicillin on the cells or penetration of the cells occurred. This was true whether the cells were centrifuged immediately after mixing with the buffer, or after 1 hour of equilibration. It may be concluded that one reason this yeast is not penicillin-sensitive is that the penicillin cannot penetrate the cell wall, and hence can have no effect.

The results obtained from similar experiments in which *S. aureus* H cells were equilibrated with penicillin solutions are summarized in figure 2. The penicillin concentration in the supernatant buffer is plotted logarithmically against the concentration in the cells, as measured by the amount of penicillin that has disappeared from the buffer. The data extend from buffer penicillin concentrations of 0.1 units per ml to 10,000 units per ml and are consistent with the hypothesis that two types of uptake occur: first, a specific uptake of approximately 0.8 units per ml that is constant and independent of the extracellular penicillin concentration and, second, a simple diffusion of penicillin into the cell so that the intracellular water has the same penicillin concentration as the extracellular water. The solid curve of figure 1, calculated on these assumptions, agrees well with the experimental data.

At higher concentrations of extracellular and intracellular penicillin, the ratio between extracellular and intracellular penicillin concentrations does not change. For every unit outside the cells there are about 0.8 units inside the cells. This would indicate that the penicillin equilibrates with all of the intracellular water, since, with these preparations, 1 ml of cells contained on the average 0.79 ml of water. If this uptake were due to adsorption on cell substance, instead of equilibration with intracellular water, it would scarcely be expected that complete equilibration with 10,000 units per ml, a 0.6 per cent penicillin solution, would occur without some evidence of saturation of the adsorbing surface.

If the experiments are extended to very low extracellular penicillin concentrations, in the neighborhood of 0.8 units per ml, complete uptake of the penicillin is observed. Due to natural variations in each batch of cells produced, and to

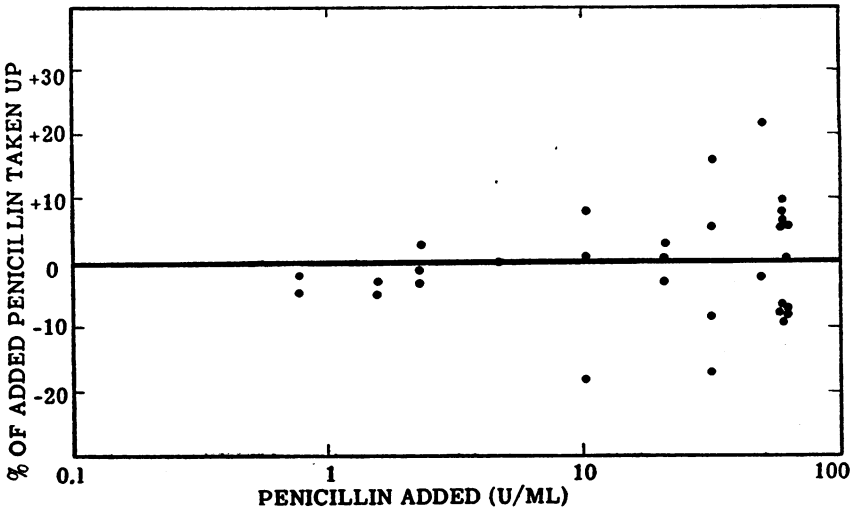

Figure 1. Penicillin uptake by cells of *S. cerevisiae.*

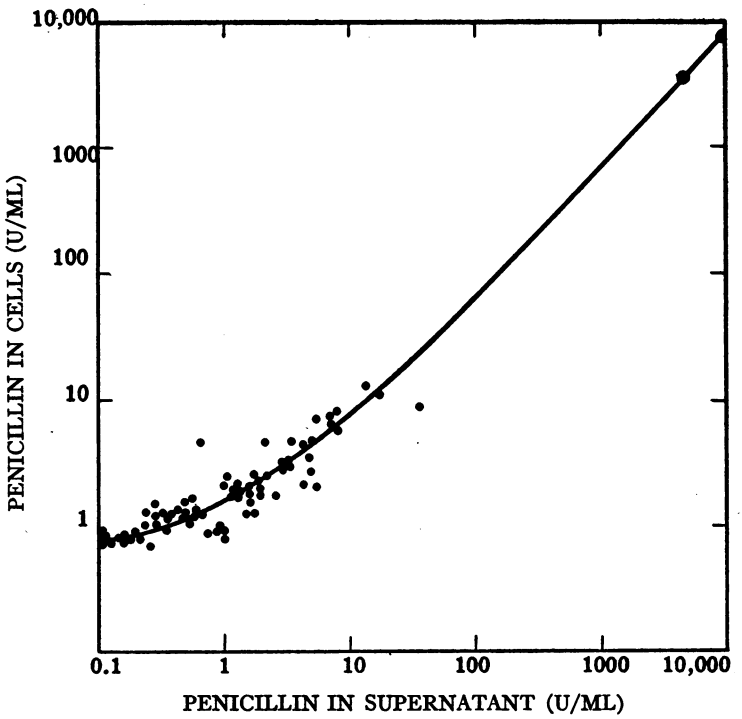

Figure 2. Penicillin uptake by cells of *S. aureus* H. The plotted points were experimentally determined; the solid curve was calculated assuming equal concentration of penicillin in intracellular and extracellular water plus specific uptake of 0.8 units per ml of cells.

assay errors, precisely 0.8 units per ml of penicillin do not always disappear from the supernatant in addition to the penicillin diffusing into the intracellular water. Experiments using cells of *S. aureus* 209P as well as *S. aureus* H in which penicillin was completely removed from the buffer solution are summarized in table 1.

The data of table 2 show that the specific uptake is not due to penicillin destruction by traces of penicillinase. Penicillin uptake is seen to be independent of the time of equilibration, which would not be true if an active penicillinase were present.

In order to determine whether bacterial cells resistant to penicillin would behave differently from sensitive cells with respect to penicillin uptake, a strain

TABLE 1

Total removal of penicillin from buffer

NO. OF EXPERIMENTS	PENICILLIN IN BUFFER	
	Before equilibration	After equilibration
	u/ml	*u/ml*
S. aureus H		
1	0.098	<0.025
6	0.187	<0.025
1	0.192	<0.025
3	0.367	<0.025
1	0.455	<0.025
2	0.90	<0.025
1	0.98	<0.025
S. aureus 209P		
1	0.1	<0.025
1	0.196	<0.025
3	0.385	<0.025
1	0.91	<0.025
1	0.983	<0.025
1	1.07	<0.025

of *S. aureus* 209P, resistant to 120 units per ml of penicillin, was tested in equilibration experiments similar to those described above. Inconclusive results were obtained. Resistant cells would at times lose the ability to absorb penicillin specifically, but would still permit the diffusion of penicillin into the intracellular water. Other data showed little or no difference between sensitive and resistant cells. These experiments were abandoned, therefore, in order to follow in greater detail the question of specific uptake of penicillin with the aid of radioactive penicillin.

Experiments equilibrating cells with radioactive penicillin were conducted for the most part under conditions similar to those with ordinary penicillin. A specific uptake was demonstrated that could now be easily detected by analyses of the cells themselves. A comparison of results obtained by radioactivity determinations and biological assay methods is presented in table 3. The two

methods give results comparable in magnitude, but the radioactivity data, because of the greater accuracy of the method, are more consistent.

The firmness with which penicillin is apparently bound to elements within the cell was demonstrated in two ways. The first type of experiment is illustrated in table 4. Cells of *S. aureus* 209P were equilibrated with various concentrations of radioactive penicillin and then washed three to five times with volumes of

TABLE 2

Effect of time on penicillin uptake (S. aureus H)

EQUILIBRATION TIME	PENICILLIN IN SUPERNATANT	
	Experiment 1	Experiment 2
hr.	*u/ml*	*u/ml*
0*	0.44	0.43
1	0.35	0.45
3	0.43	0.44

Cells were mixed with an equal volume of buffer containing 1.63 units of penicillin per ml.

* Cells centrifuged immediately after mixing.

TABLE 3

Comparison of radioactive and biological determinations of penicillin uptake

PENICILLIN ADDED TO CELL SUSPENSION	SPECIFIC UPTAKE OF PENICILLIN	
	By radioactivity determinations on cells	By *S. lutea* assay of supernatant*
u/ml	*u/ml*	*u/ml*
1.08	0.66	0.77
1.08	0.62	0.52
2.16	0.77	1.08
2.16	0.73	1.08
1.0	0.71	0.95
1.0	0.71	0.65

Ten ml of cells and 10 ml of buffer were equilibrated 10 minutes with varying amounts of radioactive penicillin. Cells were washed 3 times after equilibration.

* Calculated from assay of supernatant by difference by assuming concentration of penicillin in intracellular water is equal to concentration in supernatant.

buffer equal to the cell volume, or washed twice with volumes of buffer greatly exceeding the cell volume. None of these washing procedures caused any significant decrease in the amount of penicillin specifically taken up and firmly bound by the cell.

In the second type of experiment, which is illustrated in table 5, an attempt was made to dissociate the firmly bound penicillin from the cells. For this purpose, cells treated with radioactive penicillin and washed according to one of the procedures listed in table 4 were incubated for 10 to 30 minutes with a solution of nonradioactive penicillin containing 10,000 units per ml. Controls were

incubated under the same conditions with buffer solution replacing the concentrated penicillin solution. If the bound penicillin were in equilibrium with free penicillin within the cell it should have been replaced to some extent by nonradioactive penicillin. A comparison of control samples with those treated with

TABLE 4

Effect of extensive washing on the binding of penicillin in the cell

RADIOACTIVE PENICILLIN ADDED TO EXTRACELLULAR WATER	NO. OF WASHES	VOLUME OF EACH WASH*	RADIOACTIVE PENICILLIN BOUND TO CELLS	
			Before washing (calculated)†	After washing (analyses of cells)
u/ml			*u/ml*	*u/ml*
1.08	3	1	0.53	0.66
1.08	3	1	0.46	0.62
2.16	3	1	0.77	0.73
2.16	3	1	0.71	0.73
1.0	5	1	0.70	0.71
1.0	5	1	0.82	0.71
1.3	2	39	0.75	0.91
1.3	2	19	1.78	0.84
5.2	2	9	1.04	0.93

* Expressed as the ratio of the volume of liquid to the volume of cells.

† Difference between total penicillin in cell suspension and penicillin in the extra- and intracellular liquid.

TABLE 5

Effect of equilibrating cells containing radioactive penicillin with high concentrations of nonradioactive penicillin

RADIOACTIVE PENICILLIN ADDED TO EXTRACELLULAR WATER	INCUBATION TIME	INCUBATING SOLUTIONS*	RADIOACTIVE PENICILLIN BOUND TO CELLS	
			Before incubation with nonradioactive penicillin	After incubation with nonradioactive penicillin
u/ml	*min*		*u/ml*	*u/ml*
1.0	10	Buffer control	0.49	0.41
1.0	10	Penicillin	0.45	0.41
1.3	30	Buffer control	0.91	0.84
1.3	30	Penicillin	0.95	0.84
1.3	30	Buffer control	0.84	0.83
1.3	30	Penicillin	0.88	1.0
5.2	30	Buffer control	0.93	0.88
5.2	30	Penicillin	1.0	0.95

* Cells were washed according to one of the procedures in table 4, then incubated in buffer or in 10,000 u per ml of penicillin in amounts approximately equal to the cell volume.

concentrated penicillin demonstrates that no radioactive penicillin has been detached from the cells.

This specific binding of penicillin is small compared to the mass of cells. Calculations show that 750 molecules of penicillin are absorbed per bacterial cell.

These calculations are based on direct cell counts that indicate that 1 ml of cell paste contains an average of 1.1×10^{12} cells of *S. aureus* and specifically absorbs an average of 0.8 units of penicillin.

Pasynskiĭ and Kastorskaya (1947) calculated from their results that one bacterial cell absorbed 1,000 molecules of penicillin. Since the cells were not washed after equilibration with penicillin, the uptake measured includes penicillin present in the intracellular water. On the assumption that their cells were similar in size and specific gravity to the cells used in the present investigation, it may be calculated that approximately 300 penicillin molecules per cell were specifically absorbed. If the assumption is made that penicillin acts to block some enzymatic process in the cell essential for growth, it is easily possible that a small amount of penicillin may be effective in killing the cell. Whether this bound penicillin is actually responsible for the bactericidal activity of the antibiotic has not been determined. It is entirely possible that the observed uptake is due to a mechanism unconnected with the antibiotic activity of penicillin.

SUMMARY

Yeast cells equilibrated with equal volumes of penicillin G solutions of varying concentrations do not adsorb penicillin, nor does penicillin penetrate the cell wall.

When *Staphylococcus aureus* cells are similarly equilibrated, two types of uptake occur: (1) a specific uptake of 0.8 units per ml that is independent of the extracellular penicillin concentration, and (2) a diffusion of penicillin into the cell so that the intracellular water has the same penicillin concentration as the extracellular water. Uptake is independent of the time of equilibration.

Radioactive penicillin of high specific activity was employed to demonstrate the firmness with which the penicillin specifically absorbed is bound. Neither extensive washing nor equilibration with solutions of nonradioactive penicillin containing 10,000 units per ml released bound radioactive penicillin from the bacterial cells.

Calculations showed that 750 molecules of penicillin per bacterial cell were specifically absorbed.

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