THE RELATIONS BETWEEN BOUND PENICILLIN AND GROWTH IN STAPHYLOCOCCUS AUREUS[1]

ELEANOR A. MAASS AND MARVIN J. JOHNSON

Department of Biochemistry, University of Wisconsin, Madison, Wisconsin

Received for publication June 15, 1949

Previous experiments (Maass and Johnson, 1949) have demonstrated that if a heavy suspension of resting cells of *Staphylococcus aureus* is treated with appropriate amounts of penicillin, approximately 0.8 units of penicillin are firmly bound to each ml of cells, exclusive of the penicillin penetrating the cells by simple diffusion. It was shown by the use of radioactive penicillin that this bound penicillin cannot be displaced by extensive washing of cells, nor by incubation with high concentrations of nonradioactive penicillin. The binding of penicillin by *S. aureus* cells has recently been confirmed by Cooper and Rowley (1949). These workers also observed, apparently consistently, reduced uptake by resistant *S. aureus* cultures, whereas in our experiments uptake less than half the normal was obtained in only 7 out of 15 trials.

A more direct approach to the problem of the antibiotic action of penicillin was made in the experiments reported below by further study of the relationship of the firmly bound penicillin to the growth and metabolism of the cells. These experiments were based on the hypothesis that penicillin forms a very firm complex with some essential component of the cell. The use of radioactive penicillin made it possible to study this complex from three different points of view. First, penicillin uptake at subbacteriostatic concentrations should differ from that at higher concentrations of penicillin, if the basic essential component theory is correct. Second, it might be expected that, if the cell were actively growing during exposure to penicillin, more penicillin might be taken up because of resynthesis of the blocked essential component. Third, it is possible that resting cells, which have been exposed to penicillin and have absorbed 0.8 units per ml, when transferred to a medium suitable for growth might displace some or all of the bound penicillin from its complex before growth occurs.

METHODS

Detailed procedures for growing cells for these experiments and the techniques used for the radioactivity determinations have been described (Maass and Johnson, 1949). Cells of *S. aureus* were grown in a rich aerated medium and harvested by a Sharples centrifuge. The culture used in the following experiments was a stock culture of *S. aureus* 209P sensitive to 0.05 units per ml of penicillin. Radioactive penicillin G was obtained biosynthetically from a fer-

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This paper is based upon work sponsored in part by the Biological Department, Chemical Corps, Camp Detrick, Frederick, Maryland, under contract no. W-18-064-CM-210 with the University of Wisconsin.

mentation in a medium containing carrier-free S^{35}. The penicillin at the time of harvesting had a specific activity of 8,700 observed counts per minute per unit of penicillin. Cell suspensions or solutions tested for radioactivity were oxidized by fusion with sodium peroxide, and the sulfur was precipitated as $BaS^{35}O_4$ on flat plastic disks suitable for counting. The usual corrections for resolution time of the counter tube, for background, and for self-absorption were made. A medium containing 0.6 per cent peptone, 0.3 per cent yeast extract, 0.3 per cent beef extract, and 0.2 per cent glucose was used for growth experiments. The standard cup plate method with *S. aureus* H as the test organism was used for penicillin assays.

It was necessary in certain of these experiments to follow the increase in cell substance during growth or its constancy over long periods of incubation. The method used routinely was cell volume, measured in graduated centrifuge tubes or, when only small volumes were available, in hematocrit tubes. In either case, the suspensions were centrifuged until the observed cell volume was constant. At various times, cell weight, cell nitrogen, and turbidity were also determined. The density of dilute cell suspensions was followed by turbidity measurements in the Evelyn colorimeter using a 660-mμ filter. Dry weights were measured by weighing a dried sample of washed cells in a tared centrifuge tube. Cell nitrogen was determined on washed cells by the micro method of Johnson (1941). These methods all yielded similar data. A typical comparison is given in figure 2.

RESULTS

Uptake of penicillin at subbacteriostatic penicillin concentrations. Four-hundred-ml portions of a cell suspension containing 8 ml of cells (2 per cent by volume) were incubated in 2-liter Erlenmeyer flasks on a reciprocating shaker at 30 C with radioactive penicillin at two concentrations—0.01 unit per ml and 0.04 units per ml. These suspensions were sampled at various times, and the penicillin bound by the cells was determined. Representative data are shown in figure 1. It may be seen that, at these low concentrations, binding of penicillin was slow. This low reaction rate is not surprising in view of the fact that a penicillin solution containing 0.01 unit per ml is approximately 1.7×10^{-8} molar. It will be further noted that the rate of reaction is approximately proportional to the penicillin concentration. This is brought out in figure 1, in which the points are experimental, but the two solid curves are drawn so that, for any ordinate, the abscissa of one is four times the abscissa of the other.

This experiment was based on the assumption that the volume of cells remained relatively constant during the long period of incubation. The data of table 1, giving results of three methods of determining cell substance, indicate that this assumption was justified.

Uptake of penicillin by growing cells. Five-hundred-ml portions of medium in 2-liter Erlenmeyer flasks were inoculated with 5 ml of a 24-hour culture of *S. aureus* 209P. The cultures were incubated at 30 C on a reciprocating shaker until turbidity measurements showed that the cells were at the beginning stage of the logarithmic phase of growth. Four hours' incubation was sufficient for

this purpose. One portion of the culture was then centrifuged; the residual cells were suspended in a volume of pH 6.1 phosphate buffer equal to the volume of the cells, and treated with 0.4 units per ml of penicillin. This particular concentration was selected because it was definitely in the bacteriostatic range, and

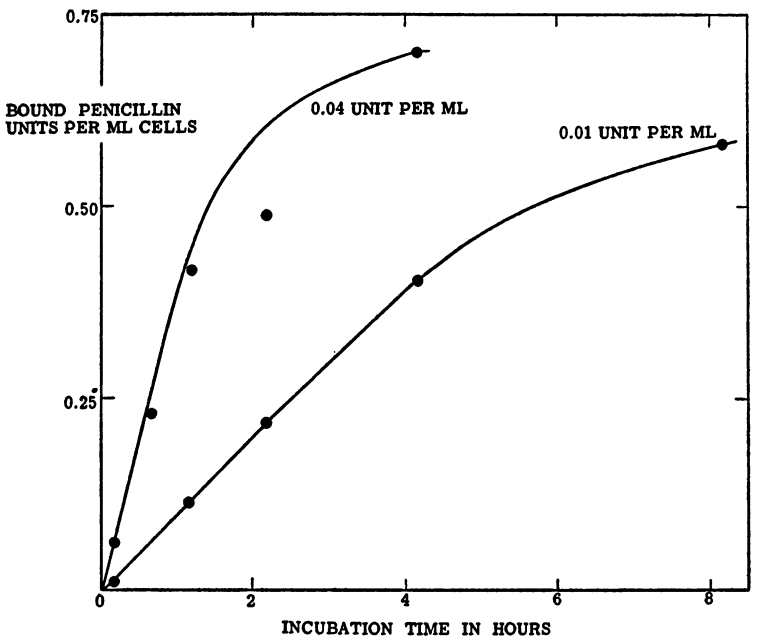

Figure 1. Penicillin uptake by cells of *S. aureus* at two subbacteriostatic concentrations of penicillin.

TABLE 1

Effect of time of incubation of cell suspensions on integrity of the cells

PENICILLIN CONCENTRATION IN MEDIUM	TIME OF INCUBATION	CELL SUBSTANCE INDICATED BY		
		Hematocrit values	Dry weight	Cell nitrogen
units/ml	*hours*	*% by volume*	*mg/ml*	*µg/ml*
0.01	0	2.0	5.85	2,250
	8	1.8	5.47	1,990
0.04	0	2.0	5.85	2,250
	4	1.6	5.85	2,120

yet low enough so that small amounts of medium contaminating the cells would not significantly affect the determination of penicillin in the cells. The penicillin bound by these cells was then determined. Data from this part of the experiments are shown in the first section of table 2. At the same time, 0.4 units per ml of penicillin were added to another portion of the culture and incubation on the shaker at 30 C was continued for 2 hours. The amount of penicillin taken up by these cells is shown in the second section of table 2. A third portion of the

culture was grown for a total of 6 hours and then treated with the same amount of penicillin. The mixture was centrifuged and the bound penicillin in the cells

TABLE 2

Penicillin uptake by growing cells

NO. OF EXPT.	CELLS GROWN 4 HR, THEN TREATED WITH PENICILLIN		CELLS GROWN 4 HR, THEN GROWN 2 HR WITH PENICILLIN		CELLS GROWN 6 HR, THEN TREATED WITH PENICILLIN	
	Penicillin bound	Cell volume*	Penicillin bound	Cell volume*	Penicillin bound	Cell volume*
	units/ml	*ml*	*units/ml*	*ml*	*units/ml*	*ml*
1	0.76	2.1	1.62	2.5	0.80	4.5
2	0.58	2.2	2.06	2.4	0.66	7.7
3	0.56	3.0	1.74	3.6	0.57	6.5

* Per 500 ml culture.

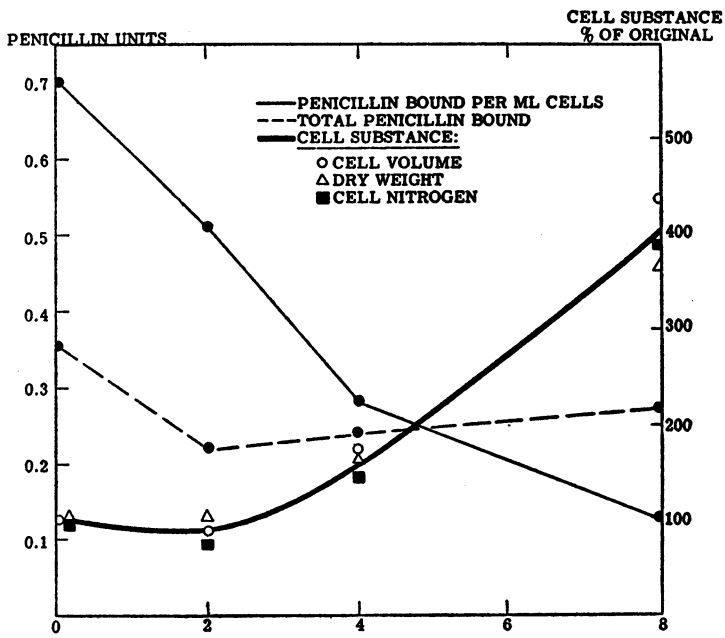

Figure 2. Displacement of bound penicillin on growth of cells in a fresh medium. Bound penicillin is plotted in terms of units per ml and also in total units. The curve for cell substance represents an average of three criteria—cell nitrogen, cell volume, and dry weight—expressed as percentage of the original value. Original values are as follows: cell nitrogen, 98 μg per ml; dry weight, 0.293 mg per ml; cell volume, 0.49 ml per 500 ml medium.

determined. These results are indicated in the third section of table 2. The total volume of cells produced in each case is also shown.

It may readily be seen from table 2 that cells growing in the presence of penicillin for 2 hours absorb two to three times more penicillin than cells grown under the same conditions and treated as resting cells with penicillin. As has previously been shown, 2-hour incubation of resting cells at penicillin concen-

trations of this order result in no greater uptake than 10-minute incubation. Cooper and Rowley (1949) have also recently reported that growing *S. aureus* cells bind more penicillin than resting cells.

Another observation that may be made from the data of table 2 is that the amount of penicillin bound by nongrowing cells is the same, whether they are grown for 4 hours and then treated with penicillin in heavy suspension or grown for 6 hours and mixed with penicillin for a short time in the original medium.

Displacement of bound penicillin by growth of penicillin-treated cells in fresh medium. Ten ml of packed cells were mixed with 10 ml of buffer containing 1 unit per ml of penicillin. After thorough washing of the cells, the penicillin absorbed by the cells was shown to be 0.70 units per ml of cells. One-half ml of these cells was then used to inoculate 500 ml of medium. The penicillin remaining on the cells was determined at 2, 4, and 8 hours. The growth of the cells was also followed during this time by the determination of cell volume in hematocrit tubes, by dry weight, and by cell nitrogen. The data of one experiment are shown in figure 2.

Consideration of the curve for penicillin bound expressed in units per ml would lead to the conclusion that there is a marked decrease in the amount of penicillin bound, and that a large displacement has occurred. However, if the total penicillin bound, regardless of cell volume, is plotted against time, it may be seen that only during the lag phase is there any decrease in the penicillin in the cells, and that this decrease is relatively slight. As the cells grow and increase in volume, the remaining penicillin is diluted by the division of the cells.

DISCUSSION

The data presented above and in the previous paper (Maass and Johnson, 1949) throw some light on the differences in penicillin action on resting, slowly growing, and rapidly growing cells. There is a considerable body of evidence in support of the hypothesis that penicillin inhibits some reaction essential to cell division, but not essential to respiration or to synthesis of many cell components (Chain and Duthie, 1945; Gale, 1948; Parker and Luse, 1948). The demonstration of extremely firm binding of a very small and relatively constant amount of penicillin by sensitive cells is an indication that some cell component, present in small amounts, combines irreversibly and rapidly with penicillin. That all of this component in the cell is combined with penicillin is indicated by the fact that the amount of combined penicillin is independent, within wide limits, of penicillin concentration and time of incubation with penicillin. If the obvious conclusion is made that this penicillin combines with a cell component essential to a reaction involved in cell division, it appears likely that this component must be a catalyst rather than a metabolite, because of the extremely small quantities involved. (Approximately 2×10^{-9} moles of penicillin are fixed by 1 ml of cells.)

Parker and Luse (1948) have suggested that the cell growth occurring when penicillin-treated cells are transferred to penicillin-free medium might occur only after resynthesis of an exhausted metabolite. The present experiments sug-

gest that resynthesis of an adequate amount of penicillin-binding component may be the chief requisite for renewed growth. From table 2 it may be seen that cells in which all the penicillin-binding component has been blocked resynthesize the new component two to three times as rapidly as cell substance. In the absence of penicillin, this resynthesis should rapidly have restored the cell to normal, permitting growth. It is interesting to note (figure 2) that the bulk of the penicillin bound by cells is not excreted when renewed growth and multiplication occurs, but remains in the daughter cells.

In view of the fact that the penicillin-binding component appears to fix penicillin even at minute concentrations and that bound radioactive penicillin does not exchange with high concentrations of free penicillin, some factor other than a dissociation constant of the bound penicillin must be responsible for the multiplication of cells at penicillin levels below the bacteriostatic concentration. The strain of *S. aureus* used in these experiments will show slight growth at 0.05 units per ml of penicillin, but no growth at 0.06 units per ml. The lag phase after transfer of the organisn is approximately 2 hours. It may be estimated from the data of figure 1 that at a penicillin concentration of 0.06 units per ml, blocking of the penicillin-binding component would be almost complete in 2 hours. It therefore appears reasonable that the lowest bacteriostatic penicillin concentration is the concentration at which blocking of the penicillin-binding component is slightly more rapid than its resynthesis by the cell.

SUMMARY

The penicillin previously reported to be firmly bound by resting *Staphylococcus aureus* cells has been found to remain largely bound during subsequent multiplication of the cells in a penicillin-free medium.

At low penicillin concentrations (0.01 to 0.04 units per ml) penicillin uptake is apparently unchanged in amount, but is much slower, many hours being required for completion of the reaction.

In a medium capable of supporting growth, cells in the presence of penicillin continue to bind penicillin, indicating more rapid synthesis of the penicillin-binding component than of cell substance.

The relation of these findings to the mode of action of penicillin is discussed.

REFERENCES

CHAIN, E., AND DUTHIE, E. S. 1945 Bactericidal and bacteriolytic action of penicillin in the *Staphylococcus*. Lancet, **248**, 652–657.

COOPER, P. D., AND ROWLEY, D. 1949 Radioactive penicillin. Nature, **163**, 480.

GALE, E. F. 1948 The nitrogen metabolism of gram-positive bacteria. Bull. Johns Hopkins Hosp., **83**, 119–175.

JOHNSON, M. J. 1941 Isolation and properties of a pure yeast polypeptidase. J. Biol. Chem., **137**, 575–586.

MAASS, E. A., AND JOHNSON, M. J. 1949 Penicillin uptake by bacterial cells. J. Bact., **57**, 415–422.

PARKER, R. F., AND LUSE, S. 1948 The action of penicillin on *Staphylococcus:* further observations on the effect of a short exposure. J. Bact., **56**, 75–81.