Penicillin: Effect on Sodium and Potassium Transport in Bacterial and Protoplast Forms of Streptococcus faecalis

Abstract. Streptococcus faecalis, *incubated in osmotically stabilized medium in the presence of 1000 units of penicillin per milliliter, accumulated rather than extruded sodium; they accumulated much less potassium than control cells did. These changes were not due to binding of sodium by cell-wall fragments of cells treated with penicillin. Because penicillin had no effect on cation concentrations in stable protoplasts and nongrowing bacterial cells, this effect appeared to be related to the production of the penicillin-induced lesions of the cell wall.*

In an effort to find out how "protoplasts" (*1*) survive in the absence of cell walls, we studied the relationship between intracellular or extracellular ionic environments (or both) and survival of bacteria after exposure to penicillin.

A previously described (*2*) strain of *Streptococcus faecalis* was grown for 18 hours in 1 liter of brain heart infusion broth (Difco). The culture was centrifuged, and the sedimented cells were resuspended, by means of a homogenizer of the Potter-Elvehjem type, in 30 ml of the supernatant. One-third of the suspension was used for determination of intracellular concentrations of sodium and potassium at zero time, and the remainder was equally divided as inoculum for two pre-warmed flasks, each containing 1 liter of the same broth with 0.5 mole of sucrose (osmotic stabilizer), 40 μmole of $MgSO_4$ and 20 g of bovine serum albumin (Armour Co.) per liter. One of the flasks also contained 1000 units of buffered potassium penicillin G (Squibb) per milliliter. The flasks were incubated at 37°C, and samples were taken at 1 and 4 hours for duplicate measurement of intracellular concentrations of sodium and potassium (*3*) and for assay of numbers of bacteria and protoplasts present.

Cytocrit tubes were filled with a concentrated suspension of microbial cells obtained by centrifugation of the samples from each culture. The tubes were then placed (*4*) in a SW 25.2 rotor and centrifuged in a Spinco Model L-2 at 20,000*g* for 30 minutes. Serum albumin-I^{131} (Abbott Laboratories) was used to measure the extracellular volume in the pellet of each sample. The pellets were ashed in nitric acid, and extracted with 0.02 percent Acationex (Scientific Products), and the sodium and potassium concentrations were determined by flame photometry.

The presence of protoplasts was determined by culturing the organisms on brain heart infusion agar containing 0.5 mole of sucrose, 40 μmole of $MgSO_4$, and 20 g of bovine serum albumin per liter, after dilution through brain heart infusion broth containing 0.5 mole of sucrose and 40 μmole of $MgSO_4$ per liter. Dilutions were also made through distilled water, and the organisms were plated on brain heart infusion agar (standard medium). The difference in the number of colonies in the two cultures represented the population of osmotically fragile protoplasts.

Cells of the stationary, 18-hour culture maintained significant concentration gradients for both sodium and potassium with respect to the medium (Table 1). When these cells were subcultured in fresh medium and incubated in the absence of penicillin, multiplication occurred and net fluxes for the first hour were in the direction of sodium extrusion and potassium accumulation. However, if penicillin was included in the culture medium, sodium net flux was reversed, and sodium was accumulated rather than extruded. In addition, although potassium net flux was, as in control cells, in the direction of accumulation, potassium was accumulated to a concentration approximately one-third that of control cells.

In the culture containing penicillin, protoplasts could be demonstrated after a 4-hour incubation with penicillin. This production of protoplasts indicated that penicillin was effective under the conditions of the experiment. The microbial population for the control (no penicillin) culture, as determined by dilution through sucrose-containing broth, was often lower than that determined by dilution through water (Table 1). Plating of samples of organisms diluted out in water on both 0.5*M* sucrose and standard plates has shown the presence of sucrose and the other additives not to be a contributing factor. The probable cause is that retention of the more viscous sucrose broth on the pipettes used for the serial dilutions is greater than that of water. This would lead to a bias error in favor of lower apparent counts on 0.5*M* sucrose dilution and plating. Therefore, the osmotically fragile microbial population for the culture containing penicillin is probably larger than that indicated by the values given in Table 1.

As a control for the citrate in Squibb buffered potassium penicillin, we repeated the experiment with unbuffered penicillin (Pfizer Laboratories). Unbuffered penicillin also caused an elevation of intracellular sodium and decreased accumulation of potassium, indicating that citrate had no effect on the observed ion fluxes.

The current view (*5*) of the mechanism of penicillin action is that penicillin prevents the completion of an intact, normal cell wall. Such a defective cell wall would probably contain an increased number of charged groups available for the binding of ions. Therefore, the observed rise in intracellular sodium in the presence of penicillin could be due to binding of sodium to the wall, which would be measured, by the methods used, as intracellular sodium. Five

Table 1. Penicillin effect on intracellular concentrations of sodium and potassium in *S. faecalis*. Standard represents microbial counts (colony-forming units, CFU) obtained by dilution through distilled water and plating on brain heart infusion agar; 0.5*M* sucrose represents microbial counts obtained by dilution through broth containing 0.5 mole of sucrose and 40 μmole of $MgSO_4$ per liter and plating on brain heart infusion agar containing 0.5 mole of sucrose, 40 μmole of $MgSO_4$, and 20 g of bovine serum albumin per liter. In addition, all plates used for counting cultures containing penicillin contained 2000 units of penicillinase (BBL) per milliliter. Sodium or potassium concentrations were measured in milliequivalents per liter of intracellular water. Medium sodium and potassium concentrations were 146 and 10.4 meq/liter, respectively. Zero time sodium and potassium values were determined on the cells used as inoculum for both flasks. Zero time microbial populations were determined on the control (no penicillin) flask only, immediately after inoculation.

Incubation time (hours)	Penicillin				No penicillin			
	Microbial population (CFU/ml) $\times 10^{-7}$		Concentration (meq/liter)		Microbial population (CFU/ml) $\times 10^{-7}$		Concentration (meq/liter)	
	Standard	0.5*M* Sucrose	Na	K	Standard	0.5*M* Sucrose	Na	K
0	13	5.8	335	52.4	13	5.8	335	52.4
1	3.9	2.3	432	181	13	5.5	73.9	455
4	0.55	3.4	538	181	116	118	219	263

300-ml centrifuge bottles were filled with an 18-hour culture, and one bottle was immediately returned to a 37°C incubator. The remaining four were centrifuged as usual, and the sedimented cells in each were homogenized in 10 ml of supernatant. The suspension from one bottle was used for determinations of intracellular sodium and potassium at zero time, and the remaining three were used to inoculate three flasks, each containing 1 liter of osmotically buffered broth, two of which also contained 1000 units of penicillin per milliliter. At the end of 1 hour's incubation at 37°C, intracellular concentrations of sodium and potassium were determined as usual on one flask with and one flask without penicillin. All of the culture of the remaining flask with penicillin was centrifuged; the sedimented cells were resuspended in distilled water, cooled on ice, and exposed to sonic vibration (full power, Biosonik, Bronwill Scientific) for ten 1-minute periods with alternating 1-minute cooling periods. The broken cells were added to the bottle of 18-hour culture still in the 37°C incubator, and intracellular concentrations of sodium and potassium were determined as usual (Table 2). The addition of broken cells from the culture with penicillin failed to increase to a significant degree the intracellular concentrations of sodium and potassium found for the 18-hour culture, and binding of sodium by an incomplete, defective cell wall produced by penicillin treatment was ruled out.

The results of two additional experiments would seem to connect the effect of penicillin on ion fluxes with its effect on cell wall synthesis. An 18-hour stationary culture was incubated further after the addition of 1000 units of penicillin per milliliter. There was no change in intracellular cation concentrations, the bacteria were not killed, and no protoplasts could be found. In the second experiment, 1000 units of penicillin per milliliter were added to a growing culture of stable *S. faecalis* protoplasts (*6*). Incubation of this organism in the presence of penicillin produced no alteration of intracellular cation concentrations. Thus, the changes in cation concentrations indicated by the experiment of Table 1 appeared to be related to cell-wall lesions induced by penicillin.

These results are at variance with those of Cooper (*7*) who noted that penicillin causes a loss of both sodium and potassium from *Staphylococcus aureus*. However, since he also reported that *S. aureus*, in the absence of penicillin, continually accumulated sodium during the rapid growth period and lost it again as growth slowed, the differences in experimental findings may be related to the species.

Table 2. The effect of broken (by sonic disruption) penicillin-treated cells on the intracellular concentrations of sodium and potassium measured for 18-hour *S. faecalis* cultures. The 18-hour culture was that used as an inoculum; the 1-hour control was incubated without penicillin; the 1-hour experimental culture was incubated with 1000 units of penicillin per milliliter.

Culture	Intracellular concentrations (meq/liter intracellular water)	
	Na	K
18-hour	298	38.3
1-hour control	123	424
1-hour experimental	464	115
18-hour culture with broken cells added	305	31.6

Using radioactive potassium, Hancock and Fitz-James (*8*) demonstrated that penicillin caused an increase in the rate of potassium efflux from *Bacillus megaterium* and that this increased efflux was completely prevented when 0.3*M* sucrose was present in the medium. In our study, net rather than unidirectional flux was studied; we found that penicillin caused a change in this flux in the presence of 0.5*M* sucrose.

The ability of *S. faecalis* in stationary phase to maintain concentration of sodium higher than that of the medium is unusual, but has been noted for *Leuconostoc citrovorum* (*9*). The concentrations of sodium and potassium found in *S. faecalis* could have been maintained by binding of the two ions to sites on or within the cells, by active inward-directed pumps for both ions, by a transmembrane potential, or by combinations of these mechanisms. Of these, neither extensive binding of ions nor an active sodium pump leading to the intracellular accumulation of sodium has been established. Consequently, it is most likely that a transmembrane potential, with the intracellular contents negative, maintained the sodium gradient. This is in contrast to stationary phase *Escherichia coli* in which no significant concentration gradient for either sodium or potassium was shown (*3*) and in which the transmembrane potential was found to be practically nonexistent (*10*). Since the potassium gradient in the stationary *S. faecalis* was larger than and in the same direction as the sodium gradient and since the two ions are of the same charge, active pumping of the potassium is indicated.

When *S. faecalis* grew in the absence of penicillin, potassium was accumulated and sodium was extruded in agreement with the results of others (*3*, *11*). One or both of these ions must be transported against its electrochemical gradient since a transmembrane potential cannot account for the different gradients of two positively charged ions. Potassium was most likely actively pumped into the cell since its concentration ratio with respect to the medium was more than 30.

Our results for a *S. faecalis*, growing in the absence of penicillin, are in qualitative agreement with those found by Zarlengo and Schultz (*11*) in that we found that the sodium-rich, potassium-poor stationary culture becomes a potassium-rich, sodium-poor organism upon growth. Those authors (*11*), however, report a higher intracellular concentration of potassium (559 meq/liter) and a much lower intracellular sodium (5 meq/liter) than we have found. Since they also find an amount of intracellular potassium (207 meq/liter) in the stationary culture which is about five times our value, we must conclude that the difference is in the strain.

Penicillin caused the accumulation rather than extrusion of sodium and less accumulation of potassium compared with control cells. The means by which these effects were brought about is unknown. Penicillin could change the transmembrane potential, inhibit active pumps extruding sodium and accumulating potassium, or do a combination of these. Neither a change in transmembrane potential alone nor an inhibition of active pumps alone could account for the changes in both ion fluxes.

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References and Notes

1. As used in this paper, "protoplast" refers to an osmotically fragile bacterial cell in which the amount of cell wall present has not been determined.
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4. Flexible Mold Material (Fry Plastics Co., Los Angeles, Calif.) was melted and poured into a Lusteroid tube (8.9 by 3.2 cm, for the SW 25.2 rotor). A stoppered cytocrit tube was held in place in the melted plastic until the plastic hardened. The level of the plastic was about halfway up the bulb portion of

the cytocrit tube. The cytocrit tube was removed, leaving the plastic cushion within the Lusteroid tube ready for use.
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6. These protoplasts were produced from *S. faecalis* by treatment with penicillin; they have been transferred 53 times on osmotically stabilized media in the presence of penicillin. Since that time, this culture has been serially transferred for more than 30 times on osmotically stabilized media in the absence of penicillin without any reversion to the bacterial form.
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12. Supported in part by grants AI 02257 and AI 03310. A preliminary report of these findings was presented at the Sixth Interscience Conference on Antimicrobial Agents and Chemotherapy, Philadelphia, Pa., 26–28 October 1966.

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Determinants of Food Intake in Obesity

Abstract. *Obese human subjects who were offered three sandwiches ate more than normal subjects. When only one sandwich was offered and additional sandwiches were available but out of sight, the obese subjects ate less than normal subjects. This result is discussed in terms of the types of cues that motivate eating for obese versus normal individuals.*

Recent work by Schachter (*1*) and his colleagues has demonstrated that the factors that govern an individual's eating behavior are related to his weight, that is, weight controlling for height. This work may be summarized by two generalizations: (i) The more an individual weighs, the less responsive he is to internal physiological cues indicative of nutritional state, and (ii) the more an individual weighs, the more responsive he is to external food- or environment-related cues.

Evidence in support of the first proposition includes a study by Stunkard and Koch (*1a*) in which a strong correspondence between extent of gastric motility and verbal reports of hunger was found for normal subjects and a much weaker correspondence for obese subjects. Schachter *et al.* (*2*) found that obese subjects ate no more food after being deprived for several hours than they did after being recently fed, while normal subjects ate much more food after they had been deprived. Goldman *et al.* (*3*) observed that obese individuals were less discomfited by enforced deprivation and altered eating schedules than normal individuals were.

The second proposition is supported by the finding of Schachter and Gross (*4*) that obese subjects ate more when they were persuaded by a speeded-up clock to believe that it was dinnertime, but this was not true for normal subjects. Nisbett (*5*) reported that overweight subjects ate far more good-tasting ice cream than ice cream adulterated with quinine, while normal subjects were less affected by the difference in taste; underweight subjects were still less affected.

If it is true that overweight individuals respond to external rather than to internal cues, it should be possible to control the amount of food they eat by varying the number of external cues that encourage eating. The most direct way to manipulate the number of external cues is simply to vary the amount of food presented to subjects. Consider the behavior we would expect of an individual who is deprived and then offered a small meal. If he is not obese, his sensitivity to internal state will motivate him to obtain more food. If it is available, he will eat more than the small amount he was offered. If he is obese, his lack of sensitivity to internal state will leave him without further motivation to eat, once he has finished the small meal. He will have eaten up all his cues, so to speak. Consider, on the other hand, the behavior we expect of an individual offered a very large meal. If he is not obese, he should leave some of it uneaten. If he is obese, he would be expected to eat most or all of the meal—in essence, he should eat until the cues are gone.

Subjects were invited to participate in an experiment involving the measurement of certain physiological variables. They were told that in order to obtain accurate base lines, it was essential that they not eat after 9:00 a.m. on the day of participation. Appointments were made for early afternoon hours so that the minimum period of deprivation was 4 hours.

The experiment was run in conjunction with one of my unpublished studies that was not concerned with eating behavior. For the purposes of that study, bogus recording electrodes were attached to the subject and he performed a "monitoring" task for approximately 30 minutes. At the end of this period the experimenter announced that the experiment was over, disengaged the subject from his electrodes, and led him into another room "to fill out some final questionnaires."

Table 1. The number of sandwiches eaten as a function of the number offered and of weight. Numerals in parentheses are numbers of subjects; MS, mean square; F, Fisher statistic.

Weight of subject	No. of sandwiches eaten when offered	
	One	Three
Underweight	1.50(10)	1.62(10)
Normal	1.96(16)	1.88(12)
Overweight	1.48(9)	2.32(12)

Analysis of variance

Source	df	MS	F
Weight (W)	2	.90	3.48*
Number offered (N)	1	1.42	5.46*
W × N	2	1.29	4.97†
Error	63	.26	

* $P < .05$. † $P = .01$.

The new experimental room contained a refrigerator, a chair, and a table on which were a bottle of soda and either one roast beef sandwich or three roast beef sandwiches. Sandwiches were wrapped in white paper. While the subject sat down, the experimenter said casually: "Since you skipped lunch for the experiment, we'd like to give you lunch now. You can fill out the questionnaires while you eat. There are dozens more sandwiches in the refrigerator, by the way. Have as many as you want." The experimenter asked the subject to check by his office on the way out, and then left, shutting the door behind him.

Several aspects of the procedure were designed to reduce possible self-consciousness on the part of overweight subjects: (i) The experimenter was absent while the subject ate, and the meal was completely private. The subject could assume that he would not be interrupted because he was to go to the experimenter's office when he was through. (ii) The subject was told that there were dozens of sandwiches in the refrigerator and could assume that if he were to take a sandwich or two it would not be missed. (iii) The subject was given no reason to assume that the experimenter had the remotest interest in how many sandwiches he ate.

Male students, in Columbia University's summer school, 25 years old or younger, whose height and weight reports indicated that they were distinctly underweight, overweight, or of normal weight, were asked to participate in the experiment. The norms published by the Metropolitan Life Insurance Com-

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