Reports

Selecting Bacterial Mutants by the Penicillin Method

Abstract. Certain improvements are described in the use of penicillin for isolating auxotrophic mutants of bacteria. By obtaining exponential growth before the penicillin is added, and by minimizing the duration of the treatment, cross-feeding is decreased and much denser populations can be screened. These modifications have made it possible to obtain, with regularity, mutants of *Escherichia coli* blocked in a desired step in arginine biosynthesis.

The use of penicillin to select auxotrophic mutants of bacteria (*1*, *2*) is based on the fact that this antibiotic kills only growing cells. In the usual practice the culture is irradiated with ultraviolet light, the mutants are allowed to become phenotypically expressed by intermediate cultivation in an enriched medium, and the cells are then washed and incubated with penicillin in minimal medium. In order to achieve sufficient killing of the parental cells it has been customary (*3*) to incubate for 6 to 24 hours; and because the auxotrophs are cross-fed by the cells that lyse during penicillin treatment, the population exposed to penicillin has been kept below a density of about 10^6 cells per milliliter. The efficiency of the method is limited by this restriction on population density, by the cross-feeding that occurs even at low population densities, and by the residual growth of mutants on stored metabolites not eliminated by washing. The last difficulty has been overcome in the multiple layer method (*4*), but the population density must still be restricted.

The present report is concerned with improvements suggested by the recent elucidation of the mode of action of penicillin. Growth in the presence of this compound ordinarily causes lysis; but in a hypertonic protective medium the cells are instead converted to protoplasts (*5*, *6*). We have accordingly added 20 percent sucrose plus extra Mg^{++} (*5*) to the penicillin selection medium in order to prevent lysis and thereby to decrease cross-feeding. In addition, it has been found possible to shorten considerably the duration of exposure to penicillin by allowing the culture to reach exponential growth in minimal medium before the penicillin is added. (This step has the additional advantage of permitting the auxotrophs present to exhaust their stores of the required growth factors.) By use of these modifications it has been found possible to screen populations at densities up to 5×10^8 cells/ml—that is, about 10^3 times greater than the densities previously feasible. The selection of rare mutants is thereby markedly improved.

We have used the following detailed technique to select arginine-requiring mutants of *Escherichia coli*. Five milliliters of a culture growing exponentially in minimal-glucose medium A (*7*) (5×10^8 to 10^9 cells/ml) is exposed to a dose of ultraviolet irradiation which reduces the viable count by a factor of 10^3 to 10^4. The irradiated sample is inoculated into 200 ml of minimal medium with 0.1 percent glucose and 100 μg of arginine per milliliter in a 2-liter flask. After overnight growth with shaking at 37°C, 5 ml (about 10^9 cells/ml) is centrifuged at 0°C and washed once with minimal medium. About one-tenth of the resuspended pellet is inoculated into 5 ml of minimal medium containing 20 percent sucrose and 0.01*M* $MgSO_4$ in a 200-ml flask. The culture is allowed to grow at 37°C with shaking until the population approximately doubles, at which time the cells are in the logarithmic phase. This step requires from 1 to 3 hours. Penicillin is then added to a final concentration of 2000 units/ml. (In one trial we obtained about the same mutant yield by using one-third this concentration.) The flask is incubated at 37°C without shaking; penicillin action is stopped by chilling and centrifuging the culture after the proper time interval. The pellet is resuspended to the previous volume with minimal medium, and samples corresponding to 10^{-2} to 10^{-4} ml are plated on solid minimal medium enriched with 0.1 percent each of casein hydrolyzate and yeast extract. We observed a better recovery of mutants by using freshly prepared plates.

The duration of exposure to penicillin is quite critical; with *E. coli* B and W it is 90 minutes. When the time was shortened to 1 hour there was insufficient killing of the wild-type cells, and when it was increasingly lengthened beyond 90 minutes there was a steady decrease in the recovery of mutants; after 3 hours none were obtained. The proper duration of exposure to penicillin can also be determined by microscopic observation, the desired time corresponding to a visible conversion of about 50 percent of the cells to protoplasts.

Wild type strains of *E. coli* including W, B, and different B × K12 recombinants have been used. In most of the cases we succeeded in isolating in a single experiment mutants blocked before ornithine, between ornithine and citrulline, and after citrulline. By irradiating mutants blocked before ornithine and using minimal medium and ornithine during the exposure to penicillin, we succeeded in superimposing a block after citrulline upon four different strains (from *E. coli* B and W). Many previous attempts to obtain such doubly blocked mutants by the classical penicillin method had been unsuccessful.

While these modifications were introduced on the basis of the expectation that the most valuable step would be prevention of the lysis of the protoplasts by use of a hypertonic medium, it now appears that the main virtue of the method lies in keeping the exposure to penicillin so brief that the penicillin-damaged prototrophs would not have time to feed the auxotrophs significantly. Thus in two experiments the absence of sucrose did not markedly affect the yield of arginine auxotrophs.

This technique has also been used successfully to obtain other amino acid and purine auxotrophs. It is reasonable to assume that it can be applied, like the usual penicillin techniques, to the selection of a large variety of auxotrophs. One must adjust the periods of preincubation and exposure to penicillin according to the generation time of the parent strain used. With the slowly growing K12 strain of *E. coli* the present method, like a previous one (*4*), yielded somewhat less satisfactory results.

So far, this method has been used primarily for selecting in a single experiment mutants with the same requirement; hence phenotypic expression is carried out in a medium supplemented with only a single growth factor. After phenotypic expression in a heavily en-

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Type manuscripts double-spaced and submit one ribbon copy and one carbon copy.

Limit the report proper to the equivalent of 1200 words. This space includes that occupied by illustrative material as well as by the references and notes.

Limit illustrative material to *one* 2-column figure (that is, a figure whose width equals two columns of text) or to *one* 2-column table or to *two* 1-column illustrations, which may consist of two figures or two tables or one of each.

For further details see "Suggestions to Contributors" [*Science* **125**, 16 (1957)].

riched medium, which results in a longer lag before resumption of growth in minimal medium, the proportion of auxotrophs among the survivors of penicillin treatment was quite low. Presumably under these conditions the prototrophs resume growth less uniformly. The present method may therefore not lend itself to the selection of a wide variety of mutants in a single experiment (*8*).

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References and Notes

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8. This work has been supported by grant No. E-2011 C2 of the U.S. Public Health Service.

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Sequential Effects of Punishment

Abstract. Punishment is found to produce a large reduction in reinforced responses when it is initially introduced. Continued exposure to punishment, however, results in substantial recovery within each hour of exposure as well as from day to day. A compensatory increase in responding occurs after the removal of punishment, even after the punishment has ceased to be effective.

The present report is concerned with the effects of punishment on behavior that is simultaneously being maintained by positive reinforcement. Previous studies (*1*) have indicated that responding is reduced so long as the punishment is in effect. The present findings reveal that the degree of suppression varies markedly during the course of the punishment process. White Carneaux pigeons, maintained at 80-percent of the weight they attained when allowed to feed ad libitum, were reinforced for 1 hour per day for responding (pecking) at an illuminated disc in accordance with a 1-minute variable-interval schedule of food reinforcement. Under this schedule, the response produces food reinforcement at varying time intervals, the average of which is 1 minute. This reinforcement procedure produces a fairly stable and uniform rate of responding which serves as a base line for evaluating the effects of punishment. This punishment was delivered immediately after every response and consisted of a brief electric shock delivered through implanted electrodes (*2*).

Figure 1 shows the effect of the addition of punishment for 23 days to the food-reinforced responses of one subject. The punishment used here is a 30-v, 60-cy/sec shock of 30-msec duration delivered through 10,000-ohms resistance in series with the subject. It can be seen that responding is reduced immediately by the initial addition of the punishment. On succeeding days the number of responses gradually increases, and recovery is complete after several days. At that time, the number of responses during punishment is equal to or greater than the number before punishment was introduced. It can be seen that, when the punishment is removed, responding increases for the first 3 days and then returns to a level approximating the prepunishment performance. It may be noted that the variable-interval schedule employed permitted the animal to receive as many food reinforcements during the punishment period as during periods without punishment as long as a low trickle of responses was made. The changes noted above of (i) a day-by-day recovery from the initial effects of punishment and (ii) a temporary increase in responding upon the elimination of punishment have both been replicated with several other subjects.

Recovery from the effects of punishment occurs not only from day to day but also within each 1-hour session. Figure 2 shows the actual cumulative response record for a different subject under more severe punishment—a shock of 10-ma intensity. Before punishment (Fig. 2, top) the rate of responding is fairly uniform throughout the hour, at about 110 responses per minute. Under punishment, however (Fig. 2, bottom), the rate of responding shows a gradual recovery throughout the hour. In the first few minutes of the punishment period, the rate of response is essentially zero, but by the end of the hour, the rate of response stabilizes at about 15 responses per minute. The absence of complete recovery from punishment here is attributable to the greater intensity of the shock used. This response record was obtained after 20 days under punishment and represents a fairly stable state. The responses show an orderly increase throughout the hour, with no increase in variability such as is generally assumed to accompany punishment. The absence of such variability is in large part attributable to the corresponding lack of variability in the shock intensity, a nonvariability achieved through the use of implanted electrodes rather than the usual electrified grid. This recovery from the initial effects of punishment within each session has characterized the behavior of all of the 14 other subjects studied, although the degree of recovery may be somewhat more or less than that seen in Fig. 2. It may be noted that this recovery does not seem to be attributable to any local tissue changes, since recovery continued when the locus of the electrodes was changed during the recovery process. Rather,

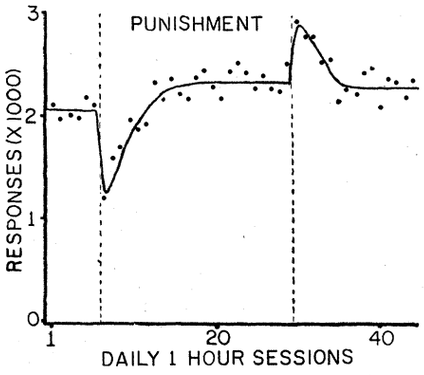

Fig. 1. Effect of the addition and removal of punishment upon the food-reinforced responses of one subject. The punishment was a brief electric shock which followed every response on the days between those represented by the vertical dashed lines. Food reinforcement was produced according to a variable-interval schedule with a mean of 1 minute on all days.

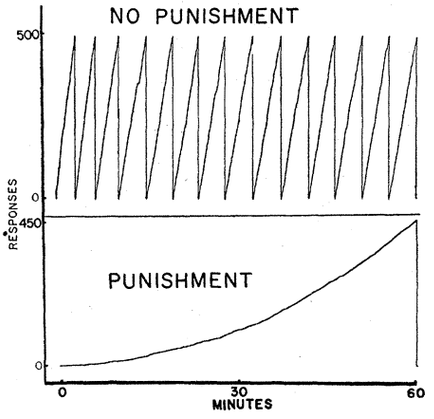

Fig. 2. (Top) Cumulative record of the food-reinforced responses of one subject prior to the addition of punishment. The food reinforcement (not indicated on the record) is produced at variable intervals, the average of which is 1 minute. The vertical lines represent the resetting of the recorder pen. (Bottom) A cumulative record of the responses for the same subject during punishment. The same schedule of food reinforcement prevails, but punishment in the form of a brief electric shock is produced by every response.