# THE RÔLE OF MUTATION AND OF SELECTION IN THE FREQUENCY OF MUTANTS AMONG MICROÖRGANISMS GROWN ON IRRADIATED SUBSTRATE

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The mutation rate in microörganisms is increased by irradiation or chemical treatment of the substrate.[1, 2] The problem of differentiating between the selection of spontaneous mutants and the induction of additional mutants is difficult, especially as the mutation rates are low and the growth rate of bacteria on treated substrate may be slower than normal. If selection is to explain the increase in frequency of mutants found in irradiated broth over that found in normal broth, it must increase the reproductive rate of mutants or inhibit it less than it inhibits the growth of normal cells. The increased number of mutants found in irradiated broth must be explained by induced mutation if selection is neutral or against the mutants.

*Experimental.*—The procedures for irradiating the medium with ultraviolet light and determining the drug resistant mutants are essentially those employed in our earlier reports.[1, 2] In addition to the stock strain of *Staphylococcus aureus* (F.D.A. #209) the first experiments reported here involved a penicillin resistant strain and a streptomycin resistant strain which arose as mutants when the stock strain was grown in irradiated broth. Identical inocula from 4-hour log phase cultures and also from 24-hour cultures (older cultures do not give consistent results) of each strain were planted in plain nutrient broth (control) and in irradiated nutrient broth. Immediately after inoculation and again after five hours, samples from each bottle were withdrawn for plating to determine the total count and the number of drug-resistant individuals in each population.

In table 1 are given the number of mutants per million organisms which are resistant to the indicated concentrations of the antibiotics, except for the figures in italics which show the total plate counts. The age of the inocula appeared to have little effect on the behavior of the organisms and the two portions of the table may be regarded as replicate experiments. Since these cultures produce a maximum of about 300 million cells per ml. under the conditions employed, it is evident that all cultures were still in the rapid growth phase at the 5-hour plating. The amount of radiation to which the broth was exposed was such that in no case did the culture growing in the irradiated broth fall behind the replicate growing in the control broth by as much as one division during the 5-hour growth period.

TABLE 1

EFFECT OF IRRADIATION OF THE BROTH ON THE GROWTH AND MUTATION RATE OF NORMAL AND DRUG-RESISTANT *S. aureus*

| | 24-HR. INOCULUM | | | | 4-HR. INOCULUM | | | |
|---|---|---|---|---|---|---|---|---|
| | CONTROL | | IRRADIATED | | CONTROL | | IRRADIATED | |
| | 0 | 5 | 0 | 5 | 0 | 5 | 0 | 5 HRS. |
| **Normal Strain** | | | | | | | | |
| Total count, millions* | *2.4* | *75* | *1.8* | *52* | *0.86* | *65* | *1.1* | *47* |
| Penicillin, 0.07 u/ml. | 13 | 19 | 17 | 344 | 7.0 | 4.6 | 9.3 | 40 |
| Streptomycin, 3.0 u./ml. | 65 | 41 | 79 | 310 | 48 | 23 | 46 | 185 |
| Streptomycin, 6.0 u./ml. | 42 | 12 | 36 | 123 | 10 | 1 | 2.8 | 40 |
| **Penicillin Resistant Strain** | | | | | | | | |
| Total count, millions* | *1.5* | *60* | *1.7* | *41* | *0.73* | *80* | *1.1* | *54* |
| Penicillin, 0.07 u./ml. | 18T | 999T | 17T | 850T | 14.5T | 1.8T | 6T | 14T |
| Streptomycin, 3.0 u./ml. | 49 | 40 | 40 | 215 | 53 | 10 | 53 | 196 |
| Streptomycin, 6.0 u./ml. | 19 | 32 | 12 | 98 | 5.5 | 6.3 | 3.5 | 21 |
| **Streptomycin Resistant Strain** | | | | | | | | |
| Total count, millions* | *0.78* | *68* | *0.82* | *53* | *1.8* | *67* | *1.6* | *60* |
| Penicillin, 0.07 u./ml. | 5.1 | 6.5 | 16 | 70 | 9.2 | 3.9 | 21 | 85 |
| Streptomycin, 3.0 u./ml. | 500T | 280T | 330T | 226T | 302T | 78T | 260T | 180T |
| Streptomycin, 6.0 u./ml. | 94T | 66T | 73T | 12.8T | 80 | 30 | 180 | 775 |

* Figures in italics are total count in millions determined by plating on nutrient agar. Other figures are the number of mutants (per million of the total count) which grew on the indicated antibiotic concentration. (T = thousand)

Yet during this period the proportion of mutants in the normal population grown in the irradiated broth increased many-fold.

It is clear that the penicillin resistant mutant resembles the normal strain in its rate of mutation to streptomycin resistance under the several test conditions. Likewise, the normal and streptomycin resistant strains have similar mutation rates to penicillin resistance. These rates, measured by rate of occurrence of organisms which grow at the indicated antibiotic concentrations, vary considerably but give a fairly consistent pattern. There is an increase in the number of resistant cells after growth on irradiated broth. However, when the changes in penicillin resistance in the penicillin resistant strain and of streptomycin resistance in the streptomycin

resistant strain under the several test conditions are compared, there is no consistent relation. In only a few instances did all the cells in the so-called resistant strains actually grow in the concentration of the antibiotic employed. Sometimes more appeared under control conditions, sometimes more from the irradiated broth. This is not in harmony with the idea that irradiated broth either conditions or selects for resistant organisms.

Selection could account for the increase in numbers of mutants in the irradiated broth only if the normal bacteria were slowed down in reproductive rate or killed, while the multiplication of the resistant mutants was speeded up or at least not retarded to the same extent. The number of times that the populations increased during the 5 hours was computed from the data on table 1. These figures for the total population and for those fractions which grew in the presence of 0.07 unit per ml. penicillin or 3 units per ml. streptomycin are recorded in table 2.

TABLE 2

INCREASE IN 5 HOURS OF THE TOTAL CELL COUNT AND OF THE MUTANTS EXPRESSED AS MULTIPLES OF THOSE PRESENT IN THE INOCULUM

| | 24-HR. INOCULUM | | | 4-HR. INOCULUM | | |
|---|---|---|---|---|---|---|
| | CONTROL | IRRADIATED | RATIO | CONTROL | IRRADIATED | RATIO |
| **Normal Strain** | | | | | | |
| Total count* | 31 (5.0) | 28 (4.8) | 0.9 | 76 (6.2) | 43 (5.4) | 0.6 |
| Penicillin mutants | 45 (5.5) | 575 (9.2) | 13 | 50 (5.7) | 185 (7.6) | 3.7 |
| Streptomycin mutants (3.0) | 20 (4.2) | 110 (6.8) | 5.5 | 36 (5.2) | 175 (7.4) | 5 |
| **Penicillin Resistant Strain** | | | | | | |
| Total count | 40 (5.4) | 24 (4.6) | 0.6 | 110 (6.8) | 49 (5.6) | 0.4 |
| Streptomycin mutants (3.0) | 33 (5.1) | 128 (7.0) | 4 | 21 (4.4) | 181 (7.5) | 8.6 |
| **Streptomycin Resistant Strain** | | | | | | |
| Total count | 87 (6.5) | 65 (6.0) | 0.7 | 37 (5.2) | 37 (5.2) | 1 |
| Penicillin mutants | 112 (6.8) | 285 (8.2) | 2.5 | 16 (4.0) | 150 (7.2) | 9.4 |

* Figures in parentheses give number of bacterial generations required to produce 5-hour population from inoculum.

Growth of the normal and the streptomycin resistant strains in irradiated broth was only slightly less than in control broth but the penicillin mutant was inhibited to a greater extent. Both the penicillin resistant mutant and the streptomycin resistant mutant were originally isolated from irradiated broth. If selection is to account for the increase in number of organisms resistant to antibiotics in irradiated broth, these mutants should have a positive selection value in irradiated broth. This is not the case. Therefore, selection fails to explain the increase in numbers of organisms resistant to antibiotics in the irradiated broth.

Table 2 shows the differential increase in resistant organisms in irradiated broth during the five-hour growth period. This is expressed both as multiplication of the original cells (normal and resistant) during that

TABLE 3

GROWTH AND MUTATIONS IN A MIXED CULTURE OF NORMAL AND MUTANT CELLS

| | HRS. | CONTROL | | IRRADIATED | |
|---|---|---|---|---|---|
| | | MUTANTS PER MILLION | TOTAL IN MILLIONS | MUTANTS PER MILLION | TOTAL IN MILLIONS |
| | | Normal Strain | | | |
| Penicillin, 0.07 u./ml. | 0 | 20 | 1.3 | 16.7 | 0.7 |
| | 2 | 15.8 | 38 | 372 | 7.8 |
| | 5 | 9.8 | 102 | 316 | 38 |
| Penicillin, 0.20 u./ml. | 0 | 0 | 1.3 | 0 | 0.7 |
| | 2 | 0 | 38 | 8.6 | 7.8 |
| | 5 | 0.4 | 102 | 4.7 | 38 |
| | | Penicillin Resistant Strain | | | |
| Penicillin, 0.07 u./ml. | 0 | 826T | 0.58 | 1000T | 0.5 |
| | 2 | 1000T | 16.3 | 992T | 1.4 |
| | 5 | 895T | 67 | 1000T | 13 |
| Penicillin, 0.20 u./ml. | 0 | 0 | 0.58 | 0 | 0.5 |
| | 2 | 0 | 16.3 | 203 | 1.4 |
| | 5 | 6.0 | 67 | 238 | 13 |
| | | Mixed Culture (0.9 Normal + 0.1 Resistant) | | | |
| Penicillin, 0.07 u./ml. | 0 | 58T | 1.7 | 48T | 1.2 |
| | 2 | 41T | 40 | 60T | 5.3 |
| | 5 | 42T | 96 | 58T | 72 |

TABLE 4

GROWTH AND MUTATIONS IN A MIXED CULTURE OF NORMAL AND MUTANT CELLS

| | HRS. | CONTROL | | IRRADIATED | |
|---|---|---|---|---|---|
| | | MUTANTS PER MILLION | TOTAL IN MILLIONS | MUTANTS PER MILLION | TOTAL IN MILLIONS |
| | | Normal Strain | | | |
| Streptomycin, 3.0 u./ml. | 0 | 25.3 | 0.8 | 29.8 | 0.8 |
| | 3 | 22.2 | 35 | 210 | 5.3 |
| Streptomycin, 10 u./ml. | 0 | 0 | 0.8 | 0 | 0.8 |
| | 3 | 0.6 | 35 | 5.1 | 5.3 |
| | | Streptomycin Resistant Strain | | | |
| Streptomycin, 3.0 u./ml. | 0 | 925T | 0.8 | 965T | 0.6 |
| | 3 | 385T | 19.4 | 429T | 3.1 |
| Streptomycin, 10 u./ml. | 0 | 210 | 0.8 | 165 | 0.6 |
| | 3 | 66.8 | 19.4 | 935 | 3.1 |
| | | Mixed Culture (0.9 Normal + 0.1 Resistant) | | | |
| Streptomycin, 3.0 u./ml. | 0 | 106T | 1.2 | 124T | 1.0 |
| | 3 | 56T | 36.0 | 104T | 7.2 |

period and as cell divisions necessary to give the net increase in numbers. The increase in number of resistant cells is of a consistently different order of magnitude from the differential increase in number of cells present in

irradiated and control media. Furthermore, the resistant mutants are similar to the normal strain in giving a differential increase in number of mutants resistant to the second antibiotic. These differences are expected if some substance in the irradiated broth causes an increase in the mutation rate. They are not in agreement with an explanation of the increase of resistant organisms based on selection.

Since it may be suggested that population dynamics in mixed cultures sometimes gives an unexpected advantage to one component, artificial mixtures of the normal and mutant organisms were prepared. The inoculum of the mixed cultures, which consisted of 9 times as many organisms from the normal strain as from the mutant strain, was planted in both irradiated and plain broth as were the requisite pure culture controls. At the time of inoculation and again 2 and 5 hours, or 3 hours later, the total count and the numbers of the mutants were determined (tables 3 and 4). It is evident that the mutants enjoyed no selective advantage in the irradiated broth. However, there was a definite and greater increase in the resistant strain of the mutants which grew in the higher concentration of antibiotics.

Another line of evidence is derived from experiments in which organisms were left in contact with the irradiated broth for varying periods of time and then washed and removed to normal broth to eliminate the possible selective action of inhibitory principles derived from the radiations. The procedure which included controls to test the effect of the washing and transferring process is described below and is outlined in the footnote of table 5.

Bottles of sterile unirradiated and irradiated nutrient broth (I A and II A) were inoculated with about $2 \times 10^6$ organisms per ml. from a log phase culture of *S. aureus*. After one-half hour a 10-ml. sample was withdrawn aseptically from each inoculated broth and placed in a sterile centrifuge tube; the bottles were incubated at 37°C. The samples were centrifuged and the sedimented organisms were washed with unirradiated broth and recentrifuged. The washed organisms were inoculated into bottles of unirradiated broth (I B and II B) from which samples were immediately withdrawn and plated in appropriate dilutions to determine the total numbers and the incidence of mutants. Further platings were made after 2- and 5-hour incubation at 37°C.

When the cultures in bottles I A and II A had been incubated $1^1/_2$ hours, another 10-ml. sample was withdrawn from each and the centrifuging and washing process performed as above. The washed organisms from the $1^1/_2$-hour cultures were inoculated into bottles of unirradiated broth (I C and II C) from which platings were made as before at 0, 2 and 5 hours after inoculation. In a like manner, a third sample was withdrawn from bottles I A and II A after they had been incubated 4 hours, but due to

TABLE 5

EFFECT OF THE TIME OF CONTACT WITH IRRADIATED BROTH ON SUBSEQUENT GROWTH AND MUTATIONS IN UNIRRADIATED BROTH

| | HOURS AFTER INOCULATION | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0 | | | 2 | | | 5 | | |
| BOTTLE | *a* | *b* | *c* | *a* | *b* | *c* | *a* | *b* | *c* |
| | | | | Experiment 1 | | | | | |
| I A | 2 | 6 | 32 | .. | ... | ... | 65 | 88 | 177 |
| II A | 3 | 4 | 22 | .. | ... | ... | 83 | 7 | 11 |
| I B | 0.6 | 16 | 48 | 6 | 65 | 158 | 83 | 46 | 57 |
| II B | 0.7 | 6 | 25 | 10 | 6 | 13 | 79 | 6 | 9 |
| I C | 0.8 | 69 | 159 | 24 | 50 | 115 | 63 | 103 | 374 |
| II C | 5.8 | 4 | 11 | 32 | 3 | 21 | 84 | 8 | 17 |
| I D | 0.8 | 100 | 186 | 12 | 82 | 262 | 63 | 35 | 145 |
| II D | 1.0 | 7 | 30 | 8 | 7 | 38 | 51 | 6 | 16 |
| | | | | Experiment 2 | | | | | |
| I A | 2 | 17 | 43 | .. | ... | ... | 55 | 300 | 102 |
| II A | 2 | 12 | 26 | .. | .... | ... | 96 | 1 | 8 |
| I B | 0.3 | 39 | 48 | 15 | 46 | 31 | 47 | 110 | 58 |
| II B | 0.4 | 12 | 31 | 19 | 8 | 6 | 55 | 2 | 8 |
| I C | 0.6 | 59 | 26 | 14 | 19 | 57 | 42 | 255 | 472 |
| II C | 2 | 15 | 20 | 33 | 1 | 6 | 87 | 4 | 14 |
| I D | 1 | 78 | 152 | 23 | 148 | 257 | .. | ... | ... |
| II D | 2 | 2 | 11 | 26 | 6 | 14 | .. | ... | ... |

*a* = total count per ml. in millions. *b* = mutants per million on 0.07 unit per ml. penicillin. *c* = mutants per million on 3 units per ml. streptomycin.

I A. Bottle of irradiated broth inoculated with log phase culture of *S. aureus*.

I B. Bottle of unirradiated broth inoculated with washed centrifuged cells which had grown in I A for $^1/_2$ hour.

I C. As I B but inoculum grown in I A for $1^1/_2$ hours.

I D. As I B but inoculum grown in I A for 4 hours.

II A, B, C, D. Same as the I series except that II A was unirradiated broth control.

growth of the culture the washed sedimented cells were diluted before inoculating into the bottles of unirradiated broth (I D and II D) so that the inoculum was about $10^6$ cells per ml. The total count and the incidence of mutants in bottles I A and II A were determined immediately after inoculation and again after 5 hours' incubation.

The results of replicate experiments are given in table 5.

It should be emphasized that the value of these tests lies in the fact that bacteria were exposed first to irradiated broth and then transferred to normal broth. The table shows that the technique of centrifuging the bacteria did not modify the results appreciably, for the two controls are essentially alike. Further, the same increase in number of mutants present is apparent in bacteria grown in irradiated broth whether or not they were centrifuged and then grown in unirradiated broth. Also this table shows that if a "high" percentage of mutants were present when the bacteria were transferred into normal broth (i.e., after four hours in irradiated broth)

this frequency was retained as the bacteria grew in normal broth. Further, if the bacteria remained in the irradiated broth for only a short period ($1^1/_2$ hours or sometimes even $^1/_2$ hour), the number of mutants present after growth in normal media increased quite markedly. The irradiated media had affected the bacteria so that the mutation rate, measured in their descendants grown in normal broth, was markedly higher after several cell divisions. Demerec[3] observed a similar effect from direct irradiation. These results are in agreement with the hypothesis that some material in the media, when utilized in the cell, causes mutations which can show in the descendants of the cell. The increase in number of mutants after transfer into normal broth is not in agreement with an explanation based on selection for resistant organisms by irradiated broth.

TABLE 6

EFFECT OF IRRADIATED BROTH ON THE OCCURRENCE OF BIOCHEMICAL MUTANTS

| | CONTROL | | | IRRADIATED | | |
|---|---|---|---|---|---|---|
| STRAIN OF *S. aureus* | NO. TESTED | MUTANTS | NO. CELLS PER MUTANT | NO. TESTED | MUTANTS | NO. CELLS PER MUTANT |
| **From mannitol + to −** | | | | | | |
| Rosenbach | 4,000 | 0 | .. | 349 | 1 | 349 |
| | 801 | 1 | 801 | 328 | 0 | .. |
| | 3,976 | 2 | 1988 | 1,064 | 3 | 355 |
| | .... | .. | .. | 3,477 | 9 | 386 |
| | 6,816 | 2 | 3408 | 10,974 | 15 | 731 |
| | 465 | 0 | .. | 4,350 | 1 | 4350 |
| Subtotal | 16,058 | 5 | 3210 | 30,542 | 29 | 1073 |
| Strain A | 1,800 | 0 | .. | 2,160 | 3 | 720 |
| Strain B | 4,600 | 1 | 4600 | 7,000 | 5 | 1400 |
| Strain C | 2,100 | 0 | .. | 4,320 | 4 | 1080 |
| Subtotal | 8,500 | 1 | 8500 | 13,480 | 12 | 1123 |
| TOTAL | 24,558 | 6 | 4093 | 44,022 | 41 | 1074 |
| **From mannitol − to +** | | | | | | |
| | 1,403 | 0 | | 3,666 | 18 | |
| | 542 | 0 | | 6,750 | 1 | |
| | 7,448 | 0 | | 4,000 | 3 | |
| | 3,666 | 0 | | .... | .. | |
| | 15,660 | 0 | | .... | .. | |
| TOTAL | 28,719 | 0 | | 14,416 | 22 | 655 |

The third series of experiments concern a different property of the organism, the ability to utilize mannitol. The normal strain of *S. aureus* used can ferment mannitol; it produces mutants which cannot. These mannitol negative organisms can in turn give rise to mannitol positive strains, presumably by reverse mutation. Table 6 shows that both the

direct and reverse mutation rate are increased by treatment with irradiated broth. The several different strains tested acted essentially alike. Grown in normal media, the combined mutation rate from mannitol + to − is one mutation in 4093; on irradiated media one in 1074; or roughly four times as great. No reverse mutation from − to + was discovered in 28,719 tested organisms grown on unirradiated broth, but 22 were found in 14,416 (1 in 655) among organisms grown in irradiated broth. The tables show the fluctuations in frequency of mutants in the several samples as would be expected from a mutation phenomenon. Although it was not proved that these − to + were reverse mutations, this theory is consistent with the situation in higher organisms. Whatever may be the truth of that matter, it is exceedingly difficult to explain how irradiated broth could select both for and against the ability to ferment mannitol.

*Summary.*—If selection is to explain the increased occurrence of mutants when *S. aureus* is grown in irradiated broth, the following conditions should be met: (1) Quantitative experiments should show that the mutants have a selective advantage over the normal strain under these conditions. This should be especially evident in a mixed culture. (2) When organisms are centrifuged from irradiated broth after a short exposure period and inoculated into unirradiated broth, the increase in the number of mutants should cease. (3) Both forward and reverse mutations should not be differentially increased by growth in irradiated broth. The results of our experiments do not fulfill any of these conditions. The results are in agreement with the hypothesis that mutations are induced by some factor in the irradiated broth.

[1] Stone, W. S., Wyss, O., and Haas, F., these PROCEEDINGS, **33**, 59–166 (1947).
[2] Wyss, O., Stone, W. S., and Clark, J. B., *J. Bact.*, **54**, 767–772 (1947).
[3] Demerec, M., these PROCEEDINGS, **32**, 36–46 (1946).

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## *ON THE AVERAGE VALUE OF ARITHMETIC FUNCTIONS*

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The purpose of this note is to introduce a new method of estimating sums of the form

$$S = \sum_{1 \leq n \leq N} f(g(n)), \tag{1}$$

where $f(n)$ is an arithmetic function such as the Euler totient function, $\phi(n)$, or the Dirichlet divisor function, $d(n)$, and $g(n)$ is either a polynomial