

# THE QUANTITATIVE ESTIMATION OF RADIATION INDUCED MUTATIONS TO STREPTOMYCIN RESISTANCE IN *ESCHERICHIA COLI*

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Received September 9, 1953

**I**N our studies of the genetic effects of radiation and radioactive isotopes, it was necessary to develop a quantitative measure of mutation rate which would remain reliable under the peculiar conditions of the requisite experiments (RUBIN 1948, 1949, 1950a; RUBIN and STEINGLASS 1949, 1950). This paper describes some observations made during the evolution of such a method.

Quantitation of the change in bacteria from streptomycin sensitivity to resistance was taken for detailed study for several reasons. Streptomycin (sulfate) is very stable under the most extreme conditions of our experiments and has the very desirable characteristic of rapidly destroying the background of sensitive organisms. Even rare mutants, therefore, can be readily demonstrated in very large populations of sensitive cells (DEMEREK 1951; RUBIN et al. 1951). Resistance to this drug (at levels exceeding 25  $\mu\text{g}$  per ml) seems to be controlled by a single genetic locus (NEWCOMBE and NYHOLM 1950) but several presumably allelic changes may be recognized (DEMEREK 1951). Thus completely resistant mutants may be obtained in the course of a single selection from a concentration of the drug which is immediately lethal to the large mass of sensitive cells. Resistant clones can then be qualitatively characterized and each mutant can be shown to have a stable type of resistance to the drug, changing only by another mutation and selection process, in a manner clearly described by BERTANI (1951).

## METHODS AND EXPERIMENTATION

The culture used in this study was *Escherichia coli* B/r, obtained from the Carnegie Institution at Cold Spring Harbor. The streptomycin was all streptomycin sulfate of one single batch purchased from the Heyden Chemical Company. Irradiation was achieved by means of a deep therapy medical X-ray unit in a manner previously described (RUBIN 1950). In the present experiments all X-radiation was done at a standard distance and setting (160 Kv and 10 ma) to provide 5000 roentgens per hour uniformly. Irradiation was always carried out at 37°C under conditions which permitted growth of the organisms (RUBIN and STEINGLASS 1950).

When aliquots of irradiated cultures are plated on agar containing streptomycin, surviving colonies (mutants) are counted with a stereoscopic microscope after at least four days of incubation at 37°C. Further incubation causes no significant increase in the numbers of mutant colonies. Controls (back-

ground mutations) of unirradiated cultures were always determined from aliquots of the inoculum used for the irradiations.

Whole culture counts on controls and irradiated cultures were done in triplicate. Three separate dilution series were made and at least three replicate plates (Petri dishes of 100 by 20 mm) of each dilution level were counted. At least nine plates were thus employed for each determination of total viable count.

All counts and mutant (streptomycin) plates were done by the multiple agar layer technique (LEDERBERG and TATUM 1946) in which the layer containing bacteria is sandwiched between two sterile layers of the same kind of agar. The counts were made on Difco Nutrient Agar, and the mutant plates on the various media to be described below. The layer plate technique was found to give greater quantitative reproducibility and to make microscopic counting easier. When contaminants appeared, they were usually confined to the surface of the agar and could be readily distinguished from the colonies of interest, which were embedded at a uniform interior level.

Usually few streptomycin-resistant cells are found in cultures plated immediately after irradiation. If a period of growth is permitted to intervene before plating, the proportion of mutants increases sharply. The characteristic pattern of increase and decline in the frequency of mutants may be seen in figure 1. A quantitation of this pattern was attempted by assaying a series of rapid subcultures in which 2 ml of a fully grown culture was added to 6 ml of sterile medium (two generations of growth permitted) and repeated as soon as growth was complete. The proportion of mutants in each subculture was determined by plating about 4 ml in from five to ten agar layer plates containing 250  $\mu$ g of streptomycin per ml. The rest of the culture was used to inoculate the subsequent subculture and for the determination of total viable count.

Since none of the mutant clones could grow faster than the non-mutants (DEMEREK 1951), the increase is clearly the result of delays in the phenotypic expression of resistance. But the sharpness of the peak and the rapidity of decline makes it evident that the loss of the more poorly surviving mutants has started even while the curve is rising. This was corroborated by an examination of the characteristics of each of the mutants found in several subculture series, which showed a disproportionately rapid decline in mutants requiring streptomycin for growth. That such a dynamic process is difficult to quantitate is shown by the examples plotted in figure 1. Although replicate irradiations gave strikingly different curves, a twofold increase in radiation dose failed to cause a proportionate increase in measurable genetic effect. In spite of careful study, no variation of the subculture technique was found capable of providing dependable quantitative data.

NEWCOMBE's (1952) adaptation of phage techniques (DEMEREK 1946) provided a key to the development of methods more suitable for the quantitation of the genetics of streptomycin resistance. As modified in the present experiments, this procedure consists of sandwiching irradiated bacteria embedded in a center agar layer between two sterile layers of the same (nutrient) agar.

After several hours of incubation at 37°C, a fourth agar layer containing streptomycin is added. The plates are then refrigerated to permit diffusion of the drug, and finally the plates are again incubated at 37°C to permit the full development of the mutant colonies.

The period of preincubation at 37°C before adding streptomycin is required to provide completion of the delayed phenotypic expression. NEWCOMBE noted that if this period was longer than six hours, the count of mutants decreased sharply. Our studies of this preincubation period showed that the time required

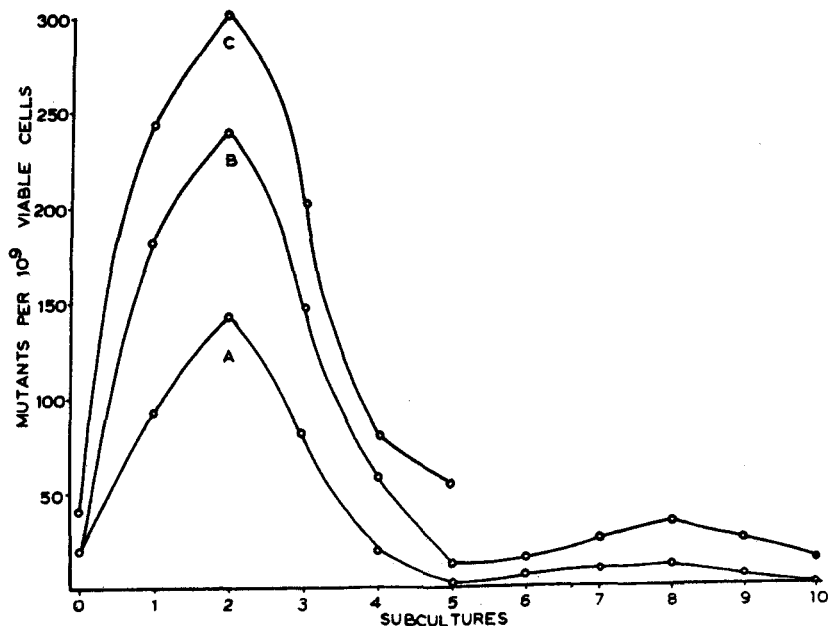


FIGURE 1.—The effect of successive subculturing on the proportion of streptomycin-resistant organisms found in irradiated cultures. The “zero point” is determined by plating aliquots of the culture immediately after irradiation. This same culture (2 ml) is then used to seed 6 ml of sterile nutrient broth. As soon as growth in this “first subculture” is complete, samples are taken for plating and subsequent subculture. Each point is the average number of colonies appearing on 10 plates containing 250  $\mu\text{g}$  of streptomycin per ml of agar. Curves A and B are from replicate cultures irradiated simultaneously for three hours at 5000 roentgens per hour. Curve C is from a culture irradiated for six hours under the same conditions.

for the maximum yield of mutants varied with the size of the aliquot of irradiated culture on the plate. When more than about  $4 \times 10^8$  viable irradiated cells were plated, the changes resulting from the variation of preincubation time were similar to those seen in the subculturing technique. No stable plateau value was ever achieved. But when fewer organisms were added, the maximum number of mutants was regularly expressed after about three hours, and there was no appreciable variation during the next seven hours (figure 2).

That the failure to obtain such a plateau is due only to an excess of viable cells was shown by adding washed unirradiated cells to small aliquots of

irradiated cultures. While living cells inhibited the formation of a plateau and diminished the mutant count, equal numbers of heat-killed cells had no such effect. The addition of various amounts of irradiated culture medium (after removal of the cells by centrifugation) also had no effect on either the number of mutants or the pattern of expression in this layer plate technique.

It may be noted in figure 2 that the maximum number of mutants detectable does not increase proportionately with the increase in culture volume. A similar observation was made by BRYSON (1951) who actually found a continuous decrease in detectable mutants as the number of irradiated cells exceeded  $10^8$  per plate. To find the lower limit of the inhibiting effect of popula-

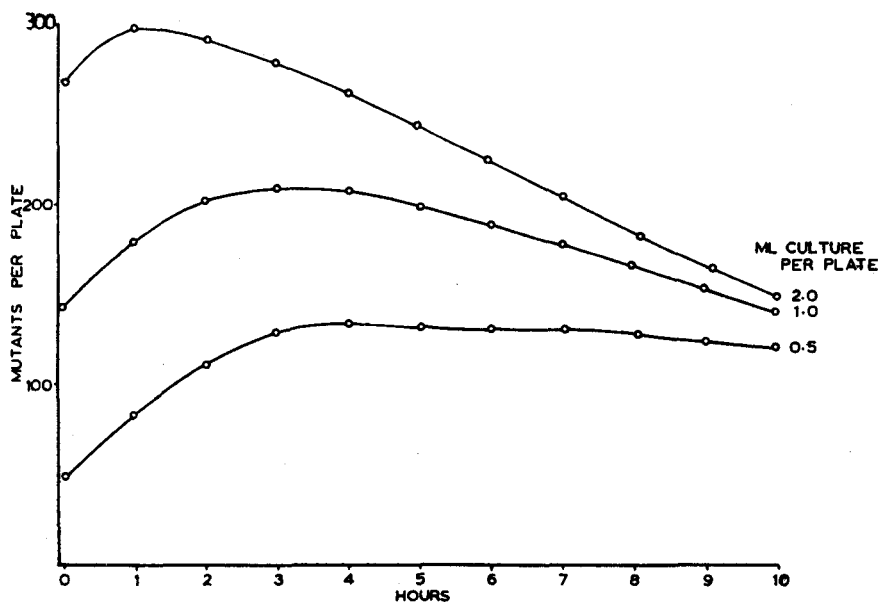


FIGURE 2.—The effect of preincubation time (before adding streptomycin) on the number of mutant colonies detectable. The culture contained  $8.2 \times 10^8$  viable cells per ml after irradiation for 4.5 hours at the rate of 5000 roentgens per hour. The streptomycin added in the fourth layer (after preincubation) was calculated to provide  $100 \mu\text{g}$  per ml after diffusion. Each point is the average of 18 replicate plates.

tion pressure we made comparisons of small culture concentrations. Complete proportionality was not achieved even when only  $10^7$  cells were plated. Smaller aliquots are difficult to test because the small number of mutants per plate introduces a large statistical error. Within practical limits, therefore, the smaller the population tested, the larger the proportion of mutants.

From figure 3 it is evident that when there are fewer than  $10^9$  cells per plate the relationship between the volume of culture plated and the number of mutants detected forms a simple linear function which may be extrapolated to zero population. The comparison of different irradiations, using this extrapolated point, shows it to be the most stable value to be found. Since the slope of the line is not constant for different irradiations, it is necessary each time

to obtain enough points in the linear region. When this is done, the agreement between experiments is as good as careful plate counting permits.

The reliability of such data depends on the size of the mutant count and the number of replicate plates. Large numbers of replicates could be prepared rapidly by pipetting the irradiated culture into a large flask of melted agar, mixing, and then dispensing the agar into several Petri dishes. For reasons of convenience in our laboratory, we usually used 500-ml flasks containing 300 ml of agar, and dispensed this into nine Petri dishes. It was found that the total mutant count per flask depends on the concentration of viable cells in the middle agar layer and not upon the volume of seeded agar per plate.

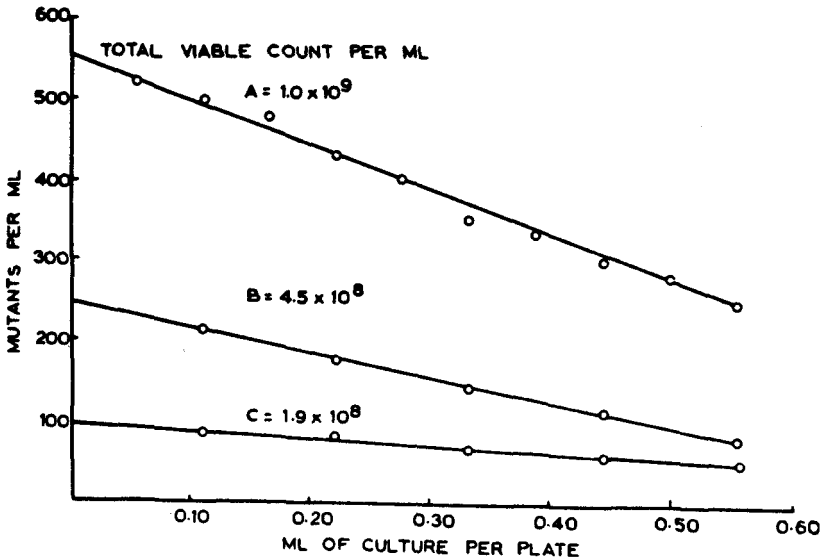


FIGURE 3.—Mutant frequency as a function of the volume of irradiated culture per plate. Three cultures were irradiated equally (5000 roentgens per hour for three hours) but at different times. By extrapolating to the point of no population effect (y axis) the mutant frequency per  $10^9$  viable cells is 550 for A, 535 for B, and 525 for C. Each point represents the average of 36 replicate plates.

In practice it was convenient to pour the contents of a flask freely (without measuring) into nine plates and then to count the nine as one unit. At least two such units of nine plates were counted for each point, and more if the mutation rate was low. The values for figure 3 were obtained in this manner. It may be noted that the average volume per plate plotted is 1 ml divided by 9; 2 ml divided by 9, etc. In this way a single pipetting of a large volume obviates the necessity for many pipettings of small volumes. The variable in figure 3 is the number of organisms per unit volume of seeded agar rather than per plate, but since these relationships were kept constant, it is equivalent to organisms per plate. Therefore, if the culture is pipetted directly onto the plate, the middle agar layer must also be carefully measured.

The preincubation time was standardized at 5 hours, a period well within

the plateau region and long before there is any tendency for the mutant count to decline. The refrigeration period (after the addition of streptomycin) usually lasted about 18 hours (overnight). No significant change in mutant count occurred by continued refrigeration as long as 96 hours. The period of preincubation in these experiments was ended very sharply by cooling the plates on a refrigerated table surface (a "Dole Plate" such as is used to keep food cold in cafeterias). The fourth layer containing streptomycin was then added to the cold Petri dishes, and the plates immediately refrigerated.

If all the layers were to be poured freely (without measurement) it was important to know the effect of streptomycin, since there was bound to be

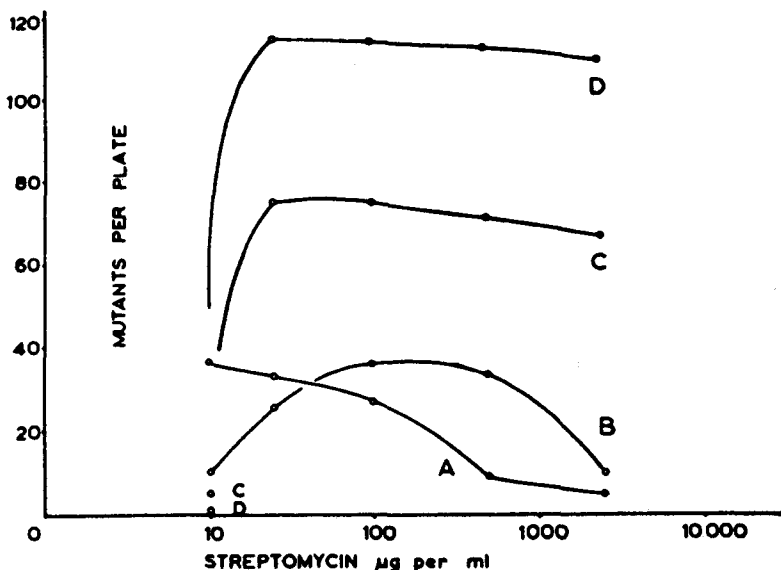


FIGURE 4.—The effect of varying the streptomycin concentration in different media on the number of mutants found in an irradiated culture. Equal aliquots of the same culture ( $2.1 \times 10^8$  viable cells per ml) were plated on: *A*—Streptomycin Assay (S.A.) Agar pH 8 in all four layers. *B*—Nutrient Agar (N.A.) pH 6.8, in all four layers. *C*—N.A. in the first three layers and B.H.I. in the fourth. *D*—S. A. in the first three layers and Brain-Heart Infusion (B.H.I.) in the fourth. Each point is the average of nine plates.

some variation in concentration. Figure 4 illustrates the effect of wide variations in streptomycin concentration on the frequency of mutants from equal aliquots from the same culture. In these experiments the volume of all the layers was carefully measured, and the concentration of streptomycin adjusted to give exactly the desired level. The periods of preincubation, refrigeration, and final incubation were carried out in the standardized 5, 18 and 96 hours respectively.

From such results it was clear that under readily attainable conditions there was little difference in the selective effect of streptomycin from 25 to 2500  $\mu\text{g}$  per ml. Therefore, the method was further standardized to use agar containing 500  $\mu\text{g}$  per ml in the fourth layer, which gives a final concentra-

tion of about 100  $\mu\text{g}$  per ml throughout the agar, after diffusion. An error in either direction of over 100 percent would make no difference in the number of mutants detectable. Numerous experiments were carried out to compare the data from plates in which all layers were poured freely and from those in which everything was carefully measured. Variation in count among replicate plates was about equal for both methods, and there was no significant difference in mutant frequency. It was thus possible to confidently avoid the multiple pipetting of small volumes and the measuring of melted agar just a few degrees above the solidification point.

It can be seen in figure 4 that the composition of the medium influences the response to streptomycin. When either Difco Streptomycin Assay Agar or Difco Nutrient Agar is used in all four layers the kind of response to variations in streptomycin concentration is less uniform than when Difco Brain Heart Infusion Agar is used for the fourth layer, and the difference in the number of mutants obtainable is quite striking. In previous experiments it had been noted that when mutants were cultured for study, more clones survived in Brain Heart Infusion Broth than in any other medium tried. In Nutrient Broth, for instance, fewer than 60 percent of the mutant clones could survive even if they had first appeared on Streptomycin Agar containing the same nutritive ingredients. In Brain Heart Infusion virtually all clones so detected did survive.

It was not feasible to use Brain Heart Infusion Agar all through a "layer plate" because of its glucose content. In the preincubation period the glucose would be fermented to produce a gassy plate, worthless in the layering technique. It was possible to use this medium in the fourth layer (which also contains the streptomycin) because the number of colonies that grew out in streptomycin is relatively small and the gas formation could then be completely inhibited by using an alkaline medium in the first three layers. By comparing different concentrations of Brain Heart Agar it was shown that its beneficial effect on the survival capacity of mutants could be achieved simply by the metabolites diffusing from a single 10-ml layer. Multiplying the concentration of the Brain Heart Infusion or increasing the volume of the fourth layer had no additional effect. Several other supplements, including nucleic acid mixtures, yeast extract, etc., were tested and found not as effective as the commercial Brain-Heart mixture.

In figure 4 it can be seen that the composition of the lower three layers also affects the final yield of mutants. It appeared advisable to use the "Assay" Agar (pH 8) in the first three layers, both to give a higher yield of mutants and also to suppress the formation of gas from the fermentation of the diffusing glucose. Other experiments (RUBIN et al. 1951) also indicated the advisability of using a medium of about pH 8 for the selection of streptomycin-resistant mutants. At more acid levels, slight changes in pH or streptomycin concentration can seriously change the effect of the drug on sensitive cells. But at a clearly alkaline level, the antibacterial and lytic effect is uniformly rapid and complete.

Another variable to be considered is the temperature of the agar in which the irradiated organisms are dispersed. It was noted that if this temperature was even slightly above 45°C, there was a serious loss of mutants. Since this temperature does not seem to greatly affect the non-mutant cells, it has been suggested that there may be a radiation reacting effect at higher temperatures (ANDERSON 1951). In any case, it was necessary to be certain that the agar was below 45°C. This was done by storing the agar for several hours in water baths or incubators held at about 45°C. The actual temperatures of pouring of the middle layers was 40–43°C. Rapid cooling was assured by spreading the plates on refrigerated table tops.

#### DISCUSSION

The extensive interest in the genetic effect of radiations, isotopes, carcinogens, etc., has stimulated the study of quantitative mutation rates in microorganisms. The desirability of using bacteria and the genetic validity of these studies have been discussed extensively but the limitations of the available techniques are not sufficiently appreciated (LEDERBERG 1948).

Until very recently the only available quantitative mutation technique in bacteria was the test for resistance to phage (DEMEREK 1946). But this involved several rather tricky techniques and remained open to a number of theoretical reservations. The long period required for complete phenotypic expression and the large proportion of zero point mutants led even DEMEREK to seek some more suitable techniques (DEMEREK 1951; DEMEREK and CAHN 1953).

Recently, quantitative mutation studies in bacteria have explored the use of genetic reversions—the loss of dependence on some metabolite or drug. The careful studies of BERTANI (1951) show the characteristic difficulties of such techniques. It is necessary to allow for the full phenotypic expression of the delayed mutations after mutagenic treatment. This would require a period of growth, and the increase of the non-mutant cells which are thereafter *not* eliminated. BERTANI solved this problem by pre-growing his cells before irradiation in a limited concentration of the substance on which they are dependent (streptomycin). He found that as he increased the concentration of streptomycin in this early growth period, he also increased the detectable number of mutants. He could not, however, reach a maximum since this would provide enough streptomycin to cause excessive growth of the non-mutants and inhibition of the mutants. This, then, is the basic difficulty of the reversion technique, there is always some background growth of the non-mutants which is hard to control while insuring complete, or even a predictable level of phenotypic expression of the mutants. DEMEREK and CAHN (1953) have devised a similar but even more complex system requiring a delicate balance between the small amount of added nutrient and the number of treated organisms plated.

The “streptomycin layer technique,” first used by NEWCOMBE and then studied by DEMEREK, permits the careful control of the factors influencing



complete expression of the mutants. NEWCOMBE did not consider the effect of population size on the pattern of genetic expression, and while DEMEREC did recognize this problem he could not solve it by means of the spraying technique which he modified from his phage methods. The present resolution of this problem shows that there is no really simple solution. Even though the pattern of genetic expression can be carefully standardized in this method, there is no way of getting around the effects of population pressure. A tempting thought is that if a higher mutation rate were available, this effect of population would be lost. But even this is probably illusory. In the course of ordinary plate counting, it is common experience to note that as the aliquot of culture on the plate increases, the apparent total viable count decreases, especially if the colonies per plate exceed 1000. This limitation of the total count on a single Petri dish was nicely described by BEALE (1948) who noted how it complicated the phage resistance technique.

If one can resign himself to the inevitable statistical problems of genetic techniques, the procedures described for obtaining a slope rather than the usual single point are really not too laborious. Actually it was frequently more trouble to obtain reliable total plate counts than the points needed to plot the mutation rate.

The statistics of this technique were improved by the use of the Brain-Heart Infusion supplement—many more mutants were thus detectable. No extensive search for more effective supplements was made, but of those tried, none proved to be better. And it was noted that almost all mutants tested could survive and grow in this medium. The use of the "free pouring" technique permitted the use of very large numbers of replicates, with the elimination of chance errors and the consequent improved statistics. The multiple layer procedure also minimizes the contamination problem.

It should be pointed out that the layer technique produced a significant number of apparent "zero point" mutants which were not seen in the serial subculture technique. This is probably related to the slow diffusion of the streptomycin to allow a period of growth, even when no preincubation period is consciously permitted. In the course of pouring of very large numbers of plates, often as many as 1500 at a time, there is also some incubation in the hot laboratory before the streptomycin layer can be added. However, since the method depends on an end point reading which is stable for several hours, the zero point reading is of no interest, except to point out that there is no disagreement with DEMEREC's findings (1951).

Although mixed culture tests as suggested by LEDERBERG (1948) were not considered feasible because of the variety of mutant types, the inverse of that test, adding unirradiated non-mutant cells, provided the same kind of information. Little selection in either direction was observed.

#### SUMMARY

The development of a quantitative genetic method is described using the mutation of *E. coli* from streptomycin sensitivity to resistance. After an ex-

amination of the variables which affect the number of mutants detectable, a standardized technique evolved which provided reproducible information about the genetic effect of radiation.

Aliquots of irradiated cultures are imbedded in the middle agar layer of a triple layer plate consisting entirely of Difco Streptomycin Assay Agar (pH 8). After a 5-hour period of incubation at 37°C, a fourth agar layer which contains 500 µg per ml of streptomycin and a supplement of Difco Brain-Heart Infusion is added. The Petri dishes are then refrigerated overnight and finally incubated again for at least four days at 37°C before the surviving colonies are counted. Several different concentrations (below  $5 \times 10^8$ ) of the culture must be treated in this manner and the value of the mutants per unit population plotted for each. The straight line which is obtained is extrapolated to the "y" axis and the intersect is taken as the mutation frequency for the culture. Non-irradiated controls, similarly treated, provide background values to be subtracted from each treatment.

#### ACKNOWLEDGMENT

It is a pleasure to acknowledge the able technical assistance of MARY F. C. PERRY and FLORENCE Z. THANASSI.

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