MUTATIONS OF BACTERIA FROM VIRUS SENSITIVITY TO VIRUS RESISTANCE[1,2]

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Received May 29, 1943

INTRODUCTION

WHEN a pure bacterial culture is attacked by a bacterial virus, the culture will clear after a few hours due to destruction of the sensitive cells by the virus. However, after further incubation for a few hours, or sometimes days, the culture will often become turbid again, due to the growth of a bacterial variant which is resistant to the action of the virus. This variant can be isolated and freed from the virus and will in many cases retain its resistance to the action of the virus even if subcultured through many generations in the absence of the virus. While the sensitive strain adsorbed the virus readily, the resistant variant will generally not show any affinity to it.

The resistant bacterial variants appear readily in cultures grown from a single cell. They were, therefore, certainly not present when the culture was started. Their resistance is generally rather specific. It does not extend to viruses that are found to differ by other criteria from the strain in whose presence the resistant culture developed. The variant may differ from the original strain in morphological or metabolic characteristics, or in serological type or in colony type. Most often, however, no such correlated changes are apparent, and the variant may be distinguished from the original strain only by its resistance to the inciting strain of virus.

The nature of these variants and the manner in which they originate have been discussed by many authors, and numerous attempts have been made to correlate the phenomenon with other instances of bacterial variation.

The net effect of the addition of virus consists of the appearance of a variant strain, characterized by a new stable character—namely, resistance to the inciting virus. The situation has often been expressed by saying that bacterial viruses are powerful "dissociating agents." While this expression summarizes adequately the net effect, it must not be taken to imply anything about the mechanism by which the result is brought about. A moment's reflection will show that there are greatly differing mechanisms which might produce the same end result.

D'HERELLE (1926) and many other investigators believed that the virus by direct action induced the resistant variants. GRATIA (1921), BURNET (1929), and others, on the other hand, believed that the resistant bacterial variants are produced by mutation in the culture prior to the addition of virus. The

[1] Theory by M. D., experiments by S. E. L.

[2] Aided by grants from the DAZIAN FOUNDATION FOR MEDICAL RESEARCH and from the ROCKEFELLER FOUNDATION.

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virus merely brings the variants into prominence by eliminating all sensitive bacteria.

Neither of these views seems to have been rigorously proved in any single instance. BURNET's (1929) work on isolations of colonies, morphologically distinguishable prior to the addition of virus, which proved resistant to the virus comes nearest to this goal. His results appear to support the mutation hypothesis for colony variants. It may seem peculiar that this simple and important question should not have been settled long ago, but a close analysis of the problem in hand will show that a decision can only be reached by a more subtle quantitative study than has hitherto been applied in this field of research.

Let us begin by restating the basic experimental finding.

A bacterial culture is grown from a single cell. At a certain moment the culture is plated with virus in excess. Upon incubation, one finds that a very small fraction of the bacteria survived the attack of the virus, as indicated by the development of a small number of resistant colonies, consisting of bacteria which do not even adsorb the virus.

Let us focus our attention on the first generation of the resistant variant—that is, on those bacteria which survive immediately after the virus has been added. These survivors we may call the "original variants." We know that these bacteria and their offspring are resistant to the virus. We may formulate three alternative hypotheses regarding them.

a. *Hypothesis of mutation to immunity*. The original variants were resistant before the virus was added, and, like their offspring, did not even adsorb it. On this hypothesis the virus did not interact at all with the original variants, the origin of which must be ascribed to "mutations" that occur quite independently of the virus. Naming such hereditary changes "mutations" of course does not imply a detailed similarity with any of the classes of mutations that have been analyzed in terms of genes for higher organisms. The similarity may be merely a formal one.

b. *Hypothesis of acquired immunity*. The original variants interacted with the virus, but survived the attack. We may then inquire into the predisposing cause which effected the survival of these bacteria in contradistinction to the succumbing ones. The predisposing cause may be hereditary or random. Accordingly we arrive at two alternative hypotheses—namely,

b_1. *Hypothesis of acquired immunity of hereditarily predisposed individuals*. The original variants originated by mutations occurring independently of the presence of virus. When the virus is added, the variants will interact with it, but they will survive the interaction, just as there may be families which are hereditarily predisposed to survive an otherwise fatal virus infection. Since we know that the offspring of the original variants do not adsorb the virus, we must further assume that the infection caused this additional hereditary change.

b_2. *Hypothesis of acquired immunity—hereditary after infection*. The original variants are predisposed to survival by random physiological variations in size, age, etc. of the bacteria, or maybe even by random variations in the

point of attack of the virus on the bacterium. After survival of such random individuals, however, we must assume that their offspring are hereditarily immune, since they do not even adsorb the virus.

These alternative hypotheses may be grouped by first considering the origin of the hereditary difference. Do the original variants trace back to mutations which occur independently of the virus, such that these bacteria belong to a few clones, or do they represent a random sample of the entire bacterial population? The first alternative may then be subdivided further, according to whether the original variants do or do not interact with the virus. Disregarding for the moment this subdivision, we may formulate two hypotheses:

1. *First hypothesis* (*mutation*): There is a finite probability for any bacterium to mutate during its life time from "sensitive" to "resistant." Every offspring of such a mutant will be resistant, unless reverse mutation occurs. The term "resistant" means here that the bacterium will not be killed if exposed to virus, and the possibility of its interaction with virus is left open.

2. *Second hypothesis* (*acquired hereditary immunity*): There is a small finite probability for any bacterium to survive an attack by the virus. Survival of an infection confers immunity not only to the individual but also to its offspring. The probability of survival in the first instance does not run in clones. If we find that a bacterium survives an attack, we cannot from this information infer that close relatives of it, other than descendants, are likely to survive the attack.

The last statement contains the essential difference between the two hypotheses. On the mutation hypothesis, the mutation to resistance may occur any time prior to the addition of virus. The culture therefore will contain "clones of resistant bacteria" of various sizes, whereas on the hypothesis of acquired immunity the bacteria which survive an attack by the virus will be a random sample of the culture.

For the discussion of the experimental possibility of distinction between these two hypotheses, it is important to keep in mind that the offspring of a *tested* bacterium which survives is resistant on either hypothesis. Repeated tests on a bacterium at different times, or on a bacterium and on its offspring, could therefore give no information of help in deciding the present issue. Thus, one has to resort to less direct methods. Two main differences may be derived from the hypotheses:

First, if the individual cells of a very large number of microcolonies, each containing only a few bacteria, were examined for resistance, a pronounced correlation between the types found in a single colony would be expected on the mutation hypothesis, while a random distribution of resistants would be expected on the hypothesis of acquired hereditary immunity. This experiment, however, is not practicable, both on account of the difficulty of manipulation and on account of the small proportion of resistant bacteria.

Second, on the hypothesis of resistance due to mutation, the proportion of resistant bacteria should increase with time, in a growing culture, as new mutants constantly add to their ranks.

In contrast to this increase in the proportion of resistants on the mutation hypothesis, a constant proportion of resistants may be expected on the hypothesis of acquired hereditary immunity, as long as the physiological conditions of the culture do not change. To test this point, accurate determinations of the proportion of resistant bacteria in a growing culture and in successive subcultures are required. In the attempt to determine accurately the proportion of resistant bacteria, great variations of the proportions were found, and results did not seem to be reproducible from day to day.

Eventually, it was realized that these fluctuations were not due to any uncontrolled conditions of our experiments, but that, on the contrary, large fluctuations are a necessary consequence of the mutation hypothesis and that the quantitative study of the fluctuations may serve to test the hypothesis.

The present paper will be concerned with the theoretical analysis of the probability distribution of the number of resistant bacteria to be expected on either hypothesis and with experiments from which this distribution may be inferred.

While the theory is here applied to a very special case, it will be apparent that the problem is a general one, encountered in any case of mutation in uniparental populations. It is the belief of the authors that the quantitative study of bacterial variation, which until now has made such little progress, has been hampered by the apparent lack of reproducibility of results, which, as we shall show, lies in the very nature of the problem and is an essential element for its analysis. It is our hope that this study may encourage the resumption of quantitative work on other problems of bacterial variation.

THEORY

The aim of the theory is the analysis of the probability distributions of the number of resistant bacteria to be expected on the hypothesis of acquired immunity and on the hypothesis of mutation.

The basic assumption of the hypothesis of acquired hereditary immunity is the assumption of a fixed small chance for each bacterium to survive an attack by the virus. In this case we may therefore expect a binomial distribution of the number of resistant bacteria, or, in cases where the chance of survival is small, a Poisson distribution.

The basic assumption of the mutation hypothesis is the assumption of a fixed small chance *per time unit* for each bacterium to undergo a mutation to resistance. The assumption of a fixed chance per time unit is reasonable only for bacteria in an identical state. Actually the chance may vary in some manner during the life cycle of each bacterium and may also vary when the physiological conditions of the culture vary, particularly when growth slows down on account of crowding of the culture. With regard to the first of these variations, the assumed chance represents the average chance per time unit, averaged over the life cycle of a bacterium. With regard to the second variation, it seems reasonable to assume that the chance is proportional to the growth rate of the bacteria. We will then obtain the same results as on the simple assump-

tion of a fixed chance per time unit, *if we agree to measure time in units of division cycles of the bacteria*, or any proportional unit.

We shall choose as time unit the average division time of the bacteria, divided by ln 2, so that the number N_t of bacteria in a growing culture as function of time t follows the equations

$$dN_t/dt = N_t, \quad \text{and} \quad N_t = N_0 e^t. \tag{1}$$

We may then define the chance of mutation for each bacterium during the time element dt as

$$adt, \tag{2}$$

so that a is the chance of mutation per bacterium per time unit, or the "mutation rate."

If a bacterium is capable of different mutations, each of which results in resistance, the mutation rate here considered will be the sum of the mutation rates associated with each of the different mutations.

The number dm of mutations which occur in a growing culture during a time interval dt is then equal to this chance (2) multiplied by the number of bacteria,[4] or

$$dm = adtN_t, \tag{3}$$

and from this equation the number m of mutations which occur during any finite time interval may be found by integration to be

$$m = a(N_t - N_0) \tag{4}$$

or, in words, to be equal to the chance of mutation per bacterium per time unit multiplied by the increase in the number of bacteria.

The bacteria which mutate during any time element dt form a random sample of the bacteria present at that time. For small mutation rates, their number will therefore be distributed according to Poisson's law. Since the mutations occuring in different time intervals are quite independent from each other, the distribution of all mutations will also be according to Poisson's law.

This prediction cannot be verified directly, because what we observe, when we count the number of resistant bacteria in a culture, is not the number of mutations which have occurred, but the number of resistant bacteria which have arisen by multiplication of those which mutated, the amount of multiplication depending on how far back the mutation occurred.

If, however, the premise of the mutation hypothesis can be proved by other means, the prediction of a Poisson distribution of the number of mutations

[4] We assume that the number of resistant bacteria is at all times small in comparison with the total number of bacteria. If this condition is not fulfilled, the total number of bacteria in this equation has to be replaced by the number of sensitive bacteria. The subsequent theoretical developments will then become a little more complicated. For the case studied in the experimental part of this paper the condition is fulfilled.

may be used to determine the mutation rate. It is only necessary to determine the fraction of cultures showing no mutation in a large series of similar cultures. This fraction p_0, according to theory, should be:

$$p_0 = e^{-m}. \tag{5}$$

From this equation the average number m of mutations may be calculated, and hence the mutation rate a from equation (4).

Let us now turn to the discussion of the distribution of the number of resistant bacteria.

The average number of resistant bacteria is easily obtained by noting that this number increases on two accounts—namely, first on account of new mutations, second on account of the growth of resistant bacteria from previous mutations. During a time element dt the increase on the first account will be, by equation (3): $adtN_t$. N_t, the number of bacteria present at time t, is given by equation (1). The increase on the second account will depend on the growth rate of the resistant bacteria. In the simple case, which we shall treat here, this growth rate is the same as that of the sensitive bacteria, and the increment on this account is ρ dt, where ρ is the average number of resistant bacteria present at time t. We have then as the total rate of increase of the average number of resistant bacteria $d\rho/dt = aN_t + \rho$ and upon integration

$$\rho = taN_t \tag{6}$$

if we assume that at time zero the culture contained no resistant bacteria.

It will be seen that the average number of resistant bacteria increases more rapidly than the total number of bacteria. Indeed the fraction of resistant bacteria in the culture increases proportionally to time. This, as pointed out in the introduction, is a distinguishing feature of the mutation hypothesis but unfortunately, as will be seen in the sequel, is not susceptible to experimental verification due to statistical fluctuations.

The resistant bacteria in any culture may be grouped, for the purpose of this analysis, into clones, taking together all those which derive from the same mutation. We may say that the culture contains clones of various age and size, calling "age" of a clone the time since its parent mutation occurred and "size" of a clone the number of bacteria in a clone at the time of observation. It is clear that size and age of a clone determine each other. If, in particular, we make the simplifying hypothesis that the resistant bacteria grow as fast as the normal sensitive strain, the relation between size and age will be expressed by equation (1), with appropriate meaning given to the symbols.

The relation implies that the size of a clone increases exponentially with its age. On the other hand, the frequency with which clones of different ages may be encountered in any culture must decrease exponentially with age, according to equations (3) and (1).

Combining these two results—namely, that clone size increases exponentially with clone age and that frequency of clones of different age decreases exponentially with clone age—we see that the two factors cancel when the

average number of bacteria belonging to clones of one age group is considered. In other words, at the time of observation we shall have, *on the average*, as many resistant bacteria stemming from mutations which occurred during the first generation after the culture was started as stemming from mutations which occurred during the last generation before observation, or during any other single generation.

On the other hand, for small mutation rates it is very improbable that any mutation will occur during the early generations of a single or of a limited number of experimental cultures. It follows that the average number of resistant bacteria derived from a limited number of experimental cultures will, probably, be considerably smaller than the theoretical value given by equation (6), and, improbably, the experimental value will be much larger than the theoretical value. The situation is similar to the operation of a (fair) slot machine, where the average return from a limited number of plays is probably considerably less than the input, and improbably, when the jackpot is hit, the return is much bigger than the input.

This result characterizes the distribution of the number of resistant bacteria as a distribution with a long and significant tail of rare cases of high numbers of resistant bacteria, and therefore as *a distribution with an abnormally high variance*. This variance will be calculated below.

For such distributions the averages derived from limited numbers of samples yield very poor estimates of the true averages. Somewhat better estimates of the averages may in such cases be obtained by omitting, in the calculation of the theoretical averages, the contribution to these averages of those events which probably will not occur in any of our limited number of samples. We may do this, in the integration leading to equation (6), by putting the lower limit of integration not at time zero, when the cultures were started, but at a certain time t_0, prior to which mutations were not likely to occur in any of our experimental cultures. We then obtain as a *likely average* r of the number of resistant bacteria in a limited number of samples, instead of equation (6),

$$r = (t - t_0)aN_t. \tag{6a}$$

It now remains to choose an appropriate value for the time interval $t - t_0$.

For this purpose we return to equation (4), in which it was stated that the average number of mutations which occur in a culture is equal to the mutation rate multiplied by the increase of the number of bacteria. Let us then choose t_0 such that up to that time just one mutation occurred, on the average, in a group of C similar cultures, or

$$1 = aC(N_{t_0} - N_0).$$

In this equation we may neglect N_0, the number of bacteria in each inoculum, in comparison with N_{t_0}, the number of bacteria in each culture at the critical time t_0. We may also express N_{t_0} in terms of N_t, the number of bacteria at the time of observation, applying equation (1):

$$N_{t_0} = N_t e^{-(t-t_0)}.$$

We thus obtain

$$\hat{t} - t_0 = \ln(N_t Ca). \tag{7}$$

Equations (6a) and (7) may be combined to eliminate $t-t_0$ and to yield a relation between the observable quantities r and N_t on the one hand and the mutation rate a on the other hand, to be determined by this equation:

$$r = aN_t \ln (N_t Ca). \tag{8}$$

This simple transcendental equation determining a may be solved by any standard numerical method. In figure 1, the relation between r and aN_t is plotted for several values of C.

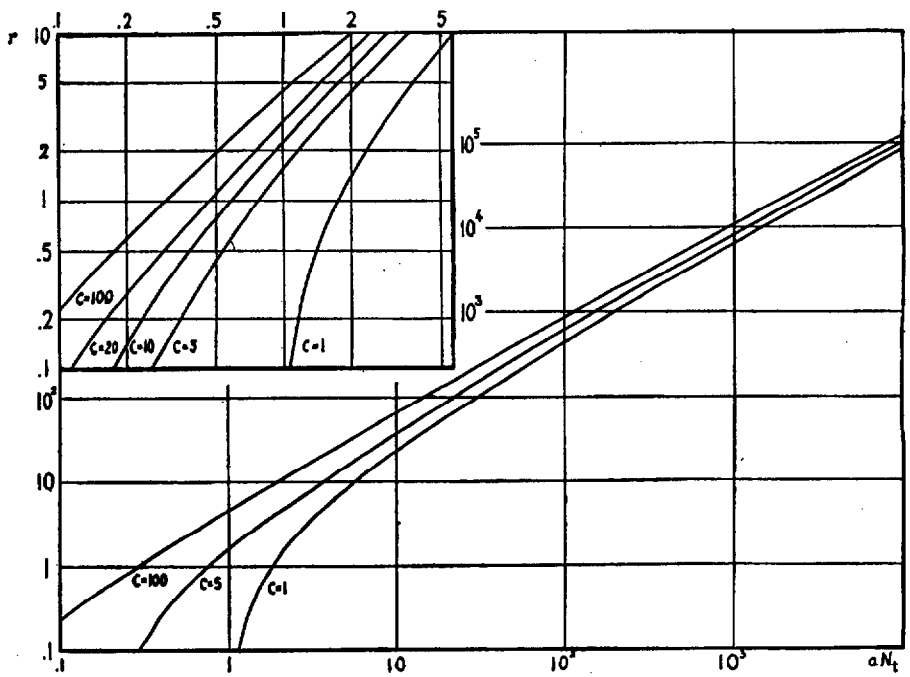

FIGURE 1.—The value of aN_t as a function of r for various values of C. The upper left hand part of the figure gives the curves for low values of aN_t and of r on a larger scale. See text.

Estimates of a obtained from equation (8) will be too high if in any of the experimental cultures a mutation happened to occur prior to time t_0. From the definition of t_0 it will be seen that this can be expected to happen in little more than half of the cases.

While we have thus obtained a relation permitting an estimate of the mutation rate from the observation of a limited number of cultures, this relation is in no way a test of the correctness of the underlying assumptions and, in particular, is not a test of the mutation hypothesis itself. In order to find such tests of the correctness of the assumption we must derive further quantitative relations concerning the distribution of the number of resistant bacteria and compare them with experimental results.

Since we have seen that the mutation hypothesis, in contrast to the hypothesis of acquired immunity, predicts a distribution of the number of resistant bacteria with a long tail of high numbers of resistant bacteria, the determination of the *variance* of the distribution should be helpful in differentiating between the two hypotheses. We may here again determine first the true variance—that is, the variance of the complete distribution—and second the likely variance in a limited number of cultures, by omitting those cases which are not likely to occur in a limited number of cultures.

The variance may be calculated in a simple manner by considering separately the variances of the partial distributions of resistant bacteria, each partial distribution comprising the resistant bacteria belonging to clones of one age group. The distribution of the total number of resistant bacteria is the resultant of the superposition of these independent partial distributions.

Each partial distribution is due to the mutations which occurred during a certain time interval $d\tau$, extending from $(t-\tau)$ to $(t-\tau+d\tau)$. The average number of mutations which occurred during this interval is, according to equation (3),

$$dm = aN_\tau d\tau = aN_t e^{-\tau} d\tau. \tag{9}$$

These mutations will be distributed according to Poisson's law, so that the variance of each of these distributions is equal to the mean of the distribution. We are however not interested in the distribution of the number of mutations but in the distribution of the number of resistant bacteria which stem from these mutations at the time of observation—that is, after the time interval τ. Each original mutant has then grown into a clone of size e^τ. The distribution of the resistant bacteria stemming from mutations occurred in the time interval $d\tau$ has therefore an average value which is e^τ times greater than the average number of mutations, and a variance which is $e^{2\tau}$ times greater than the variance of the number of mutations. Thus we find for the average number of resistant bacteria:

$$d\rho = aN_t d\tau,$$

and for the variance of this number

$$\mathrm{var}_{d\rho} = aN_t e^\tau d\tau.$$

From this variance of the partial distribution, the variance of the distribution of all resistant bacteria may be found simply by integrating over the appropriate time interval—that is, either from time t to time o (τ from o to t), if the true variance is wanted, or from time t to time t_0 (τ from o to $t-t_0$), if the likely variance in a limited number of cultures is wanted. In the first case we obtain:

$$\mathrm{var}_\rho = aN_t(e^t - 1). \tag{10}$$

In the second case we obtain:

$$\mathrm{var}_r = aN_t\left[e^{(t-t_0)} - 1\right]. \tag{10a}$$

Substituting here the previously found value of $(t-t_0)$ and neglecting the second term in the brackets, we obtain:

$$\text{var}_r = Ca^2N_t^2. \tag{11}$$

Comparing this value of the likely variance with the value of the likely average, from equation (8), we see that the ratio of the standard deviation to the average is:

$$\sqrt{\text{var}_r}/r = \sqrt{C}/\ln (N_tCa). \tag{12}$$

It is seen that this ratio depends on the logarithm of the mutation rate and will consequently be only a little smaller for mutation rates many thousand times greater than those considered in the experiments reported in this paper.

In the beginning of this theoretical discussion we pointed out that the hypothesis of acquired immunity leads to the prediction of a distribution of the number of resistant bacteria according to Poisson's law, and therefore to the prediction of a variance equal to the average. On the other hand, if we compare the average, equation (8), with the variance, equation (11), (not, as above, with the square root of the variance), we obtain

$$\text{var}_r = rN_tCa/\ln (N_tCa). \tag{12a}$$

Equation (12a) shows that the likely ratio between variance and average is much greater than unity on the hypothesis of mutation, if (N_tCa), the total number of mutations which occurred in our cultures, is large compared to unity.[5]

It is possible to carry the analysis still further and to evaluate the higher moments of the distribution function of the number of resistant bacteria, or even the distribution function itself. The moments are comparatively easy to obtain, while the calculation of the distribution function involves considerable

[5] In some of the experiments reported in the present paper we did not determine the total number of resistant bacteria in each culture, but the number contained in a small sample from each culture. In these cases the variance of the distribution of the number of resistant bacteria will be slightly increased by the sampling error. The proper procedure is here first to find the average number of resistant bacteria per culture by multiplying the average per sample by the ratio

$$\frac{\text{volume of culture}}{\text{volume of sample}}; \tag{13}$$

second, to evaluate the mutation rate with the help of equation (8); third, to figure the likely variance for the cultures by equation (11); fourth, to divide this variance by the square of the ratio (13) to obtain that part of the variance in the samples which is due to the chance distribution of the mutations. The experimental variance should be greater than this value, on account of the sampling variance. The sampling variance is in all our cases only a small correction to the total variance, and it is sufficient to use its upper limit, that of the Poisson distribution, in our calculations. Consequently, when comparing the experimental with the calculated values, we first subtract from the experimental value the sampling variance, which we take to be equal to the average number of resistant bacteria.

mathematical difficulties. An approximation to the beginning of the distribution function—that is, to its values for small numbers of resistant bacteria—may be obtained by grouping mutations according to the bacterial generation during which they occurred. For instance, the probability of obtaining seven resistant bacteria may be broken down into the sum of the following alternative events: (a) seven mutations during the last generation; (b) three mutations during the last generation and two mutations one generation back; (c) three mutations during the last generation and one mutation two generations back; (d) one mutation during the last generation and three mutations one generation back; (e) one mutation during the last generation, one mutation one generation back and one mutation two generations back.

The probability of each of these events depends only on the mutation rate and on the final number of bacteria.

The grouping of mutations according to the bacterial generation during which they occurred, and the assumption that the bacteria increase in simple geometric progression, simplify the calculation sufficiently to permit numerical computation. On the other hand, the classes with two, four, eight, etc., mutants are artificially favored by this procedure, so that a somewhat uneven distribution results, with too high values for two, four, eight, etc., resistant bacteria (see fig. 2).

MATERIAL AND METHODS

The material used for our experimental study consisted of a bacterial virus α and of its host, *Escherichia coli* B (DELBRÜCK and LURIA 1942). Secondary cultures after apparently complete lysis of B by virus α show up within a few hours from the time of clearing. They consist of cells which are resistant to the action of virus α, but sensitive to a series of other viruses active on B. The resistant cells breed true and can be established easily as pure cultures. No trace of virus could be found in any pure culture of the resistant bacteria studied in this paper. The resistant strains are therefore to be considered as non-lysogenic.

Tests were made to see whether the resistance to virus α was a stable character of the resistant strains. In the first place, it was found that virus α is not appreciably adsorbed by any of the resistant strains. In the second place, when a certain amount of virus α is mixed with a growing culture of a resistant strain, no measurable increase of the titer of virus α occurs over a period of several hours. This is a very sensitive test for the occurrence of sensitive bacteria, and its negative result for all resistant strains shows that reversion to sensitivity must be a very rare event.

Morphologically at least two types of colonies of resistant bacteria may be distinguished. The first type of colony is similar to the type produced by the sensitive strain both in size and in the character of the surface and of the edge. The second type of colony is much smaller and translucent. The difference in colony type is maintained in subcultures. Microscopically the bacteria from these two types of colonies are indistinguishable. They also do not differ

from each other or from the sensitive strain in their fermentation reactions on common sugars and in the characteristics of their growth curves in nutrient broth. In particular, the lag periods, the division times during the logarithmic phase of growth, and the maximum titers attained are identical for the sensitive strain and for the two variants. Both variants, therefore, fulfill the requirements for the applicability of the theory developed above.

In the presentation of our experimental results we have lumped the counts of the two types of colonies together, because: (1) theoretically, this is equivalent to summing the corresponding mutation rates; (2) experimentally, we are not certain whether each of these types does not actually comprise a diversity of variants; (3) experimentally, no correlation appeared to exist between the occurrence of these variants, which shows the independence of the causes of their occurrence.

Cultures of B were grown either in nutrient broth (containing .5 percent NaCl) or in an asparagin-glucose synthetic medium. In the latter, the division time during the logarithmic phase of growth was 35 minutes, as compared with 19 minutes in broth. In synthetic medium, the acidity increased during the time of incubation from pH 7 to pH 5.

In cultures of strain B, between 10^{-8} and 10^{-6} of the bacteria are found usually to give colonies resistant to the action of virus α when samples of such cultures are plated with large amounts of virus. In order to be reasonably certain that the resistant bacteria found in the test had not been introduced into the test culture with the initial inoculum, the test cultures were always started with very small inocula, containing between 50 and 500 bacteria from a growing culture. Thus any resistant bacterium found at the moment of testing (when the culture contains between 10^8 and 5×10^9 bacteria/cc) must be an offspring of one of the sensitive bacteria of the inoculum.

All platings were made on nutrient agar plates. The plating experiments for counting the number of resistant bacteria in a liquid culture of the sensitive strain were done by plating either a portion or the entire culture with a large amount of virus α. The virus was plated first, and spread over the entire surface of the agar. A few minutes later the bacterial suspension to be tested was spread over the central part of the plate, leaving a margin of at least one centimeter. Thus all bacteria were surrounded by large numbers of virus particles.

Microscopic examination of plates seeded in this manner showed that lysis takes place very quickly; only bacteria which at the time of plating were in the process of division may sometimes complete the division. The resistant colonies which appear after incubation are therefore due to resistant bacterial cells present at the time of plating.

The total number of bacteria present in the culture to be tested was determined by colony counts in the usual manner.

The resistant colonies of the large type appear after 12–16 hours of incubation, the colonies of the small type appear after 18–24 hours, and never reach half the size of the former ones. Counts were usually made after 24 and 48 hours.

EXPERIMENTAL

A Test of the Reliability of the Plating Method

In our experiments we wanted to study the fluctuations of the numbers of resistant bacteria found in cultures of sensitive bacteria. It was therefore necessary to show first that the method of testing did not involve any unrecognized variables, which caused the number of resistant colonies to vary from plate to plate or from sample to sample.

Therefore, parallel platings were made using a series of samples from the same bacterial culture. If our plating method is reliable, fluctuations should in this arrangement be due to random sampling only, and the variance from a series of such samples should be equal to the mean.

Table 1 gives the results of three such experiments. It will be seen that in

TABLE 1

The number of resistant bacteria in different samples from the same culture.

SAMPLE NO.	EXP. NO. 10a RESISTANT COLONIES	EXP. NO. 11a RESISTANT COLONIES	EXP. NO. 3 RESISTANT COLONIES
1	14	46	4
2	15	56	2
3	13	52	2
4	21	48	1
5	15	65	5
6	14	44	2
7	26	49	4
8	16	51	2
9	20	56	4
10	13	47	7
mean	16.7	51.4	3.3
variance	15	27	3.8
χ^2	9	5.3	12
P	.4	.8	.2

all three cases variance and mean agree as well as may be expected. There is therefore no reason to assume that the method of sampling or plating introduces any fluctuations into our results besides the sampling error.

Fluctuations of the Number of Resistant Bacteria in Samples from a Series of Similar Cultures

As pointed out in the introduction and in the theoretical part, the hypothesis of acquired immunity and the hypothesis of mutation lead to radically different predictions regarding the distribution of the number of resistant bacteria in a series of similar cultures. The hypothesis of acquired immunity predicts a variance equal to the average, as in sampling, while the mutation hypothesis predicts a much greater variance.

Series of five to 100 cultures were set up in parallel with small equal inocula, and were grown until maximum titer was reached. Three kinds of cultures

were used—namely: (1) 10.0 cc aerated broth cultures; (2) .2 cc broth cultures; (3) .2 cc synthetic medium cultures.

The results of all tests for the number of resistant bacteria are summarized in table 2 and table 3.

TABLE 2

The number of resistant bacteria in series of similar cultures.

EXPERIMENT NO.	1	10	11	15	16	17	21a	21b
Number of cultures	9	8	10	10	20	12	19	5
Volume of cultures, cc	10.0	10.0	10.0	10.0	.2*	.2*	.2	10.0
Volume of samples, cc	.05	.05	.05	.05	.08	.08	.05	.05
Culture No.								
1	10	29	30	6	1	1	0	38
2	18	41	10	5	0	0	0	28
3	125	17	40	10	3	0	0	35
4	10	20	45	8	0	7	0	107
5	14	31	183	24	0	0	8	13
6	27	30	12	13	5	303	1	
7	3	7	173	165	0	0	0	
8	17	17	23	15	5	0	1	
9	17		57	6	0	3	0	
10			51	10	6	48	15	
11					107	1	0	
12					0	4	0	
13					0		19	
14					0		0	
15					1		0	
16					0		17	
17					0		11	
18					64		0	
19					0		0	
20					35			
Average per sample	26.8	23.8	62	26.2	11.35	30	3.8	48.2
Variance (corrected for sampling)	1217	84	3498	2178	694	6620	40.8	1171
Average per culture	5360	4760	12400	5240	28.4	75	15.1	8440
Bacteria per culture	3.4×10^{10}	4×10^{10}	4×10^{10}	2.9×10^{10}	5.6×10^{8}	5×10^{8}	1.1×10^{8}	3.2×10^{10}
Mutation rate	1.8×10^{-8}	1.4×10^{-8}	4.1×10^{-8}	2.1×10^{-8}	1.1×10^{-8}	3.0×10^{-8}	3.3×10^{-8}	3.0×10^{-8}
Standard deviation / Average, exp.	1.3	.39	.95	1.8	2.3	2.7	1.7	.71
Standard deviation / Average, calc.	.35	.33	.33	.37	.94	.67	1.04	.26

* Cultures in synthetic medium.

It will be seen that in every experiment the fluctuation of the numbers of resistant bacteria is tremendously higher than could be accounted for by the sampling errors, in striking contrast to the results of plating from the same culture (see table 1) and in conflict with the expectations from the hypothesis of acquired immunity.

We want to see next whether these results fit the expectations from the hypothesis of mutation. We must therefore compare the experimental results with the relations developed in the theoretical part, keeping in mind that the theory contains several simplifying assumptions.

First we can compare, according to equation (12), the experimental and the calculated values of the ratio between the standard deviation and the average of the numbers of resistant bacteria. These ratios are included in tables 2 and 3. It is seen that the experimental and theoretical values are reasonably close.

However, in all but one case the experimental ratio is greater than the value calculated from the theory—that is, the variability is even greater than predicted.

TABLE 3

Distribution of the numbers of resistant bacteria in series of similar cultures.

EXPERIMENT NO.	22		23	
Number of cultures	100		87	
Volume of cultures, cc	.2*		.2*	
Volume of samples, cc	.05		.2	
	Resistant bacteria	*Number of cultures*	*Resistant bacteria*	*Number of cultures*
	0	57	0	29
	1	20	1	17
	2	5	2	4
	3	2	3	3
	4	3	4	3
	5	1	5	2
	6– 10	7	6– 10	5
	11– 20	2	11– 20	6
	21– 50	2	21– 50	7
	51– 100	0	51– 100	5
	101– 200	0	101– 200	2
	201– 500	0	201– 500	4
	501–1000	1	501–1000	0
Average per sample	10.12		28.6	
Variance (corrected for sampling)	6270		6431	
Average per culture	40.48		28.6	
Bacteria per culture	2.8×10^8		2.4×10^8	
Mutation rate	2.3×10^{-8}		2.37×10^{-8}	
Standard deviation / Average, exp.	7.8		2.8	
Standard deviation / Average, calc.	1.5		1.5	

* Cultures in synthetic medium.

A part of this discrepancy may be accounted for by the fact that the time t_0, mutations occurring prior to which were disregarded by the theory, was chosen in such a manner that on the average one mutation would occur prior to time t_0. This mutation, if it occurs, will of course tend to increase the variance, and in some of the experiments the high value of the experimental variance can be traced directly to one exceptional culture in which a mutation had evidently occurred several generations prior to time t_0. Unfortunately, there is no general criterion by which one might eliminate such cultures from the statistical analysis, because, in a culture with an exceptionally high count of resistant bacteria, these do not necessarily stem from one exceptionally early mutation, but may also be due to an exceptionally large number of mutations after time t_0.

There may also be other reasons why the observed variances are higher than the expected ones. First of all, the simplifying assumption that the mutation

rate per bacterial generation is independent of the physiological state of the bacteria may be too simple. If the mutation rate is higher for actively growing bacteria than for bacteria near the saturation limit of the cultures, early mutations and big clone sizes will be favored, and therefore higher variations of the numbers of resistant bacteria can be expected. Second, the assumption of a sudden transition from sensitivity to resistance may also be too simple. It is conceivable that the character "resistance to virus" may not fully develop in the bacterial cell in which the mutation occurs, but only in its offspring, after one or more generations. However, if this were the case, cultures with only one or two resistant bacteria should be relatively rare. The last experiment listed in table 3, in which the entire cultures were plated, shows a rather high proportion of cultures with only one resistant bacterium. This seems to show that the

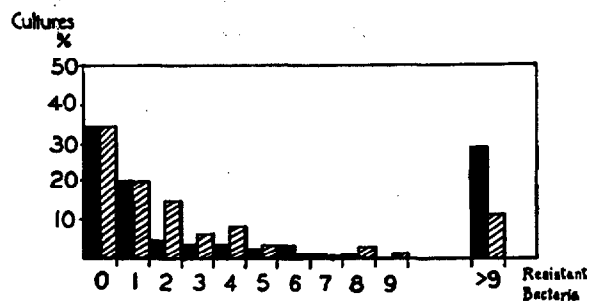

FIGURE 2.—Experimental (Experiment No. 23) and calculated distributions of the numbers of resistant bacteria in a series of similar cultures. Solid columns: experimental. Cross-hatched columns: calculated.

character "resistance to virus" in general does come to expression in the bacterial cell in which the corresponding mutation occurred, as assumed by the theory.

Another way of comparing the experimental results with the theory is to compare the experimental distribution of resistant bacteria with the approximate distribution calculated by the method outlined at the end of the theoretical part. The theoretical distribution has to be calculated from the average number of mutations per culture given by equation (5). Only experiments where the whole culture is tested can therefore be used for such a comparison. This method tests the fitting of the expectations for small numbers of resistant bacteria, in contrast to the comparison of the standard deviations, which involves predominantly the cultures with high numbers of resistant bacteria.

Figure 2 shows the experimental and calculated distributions for Experiment No. 23; the cultures with more than nine resistant bacteria are lumped together in one class, since the distribution has not been calculated for values higher than nine.

It is seen that the fitting for small values is satisfactory. In particular, the

number of cultures with one resistant bacterium very closely fits the expectation. The classes with two, four, eight, etc., resistant bacteria are bound to be favored in the theoretical distribution, as explained in the theoretical part.

The results shown in figure 2 also confirm the assumption that the discrepancy between experimental and calculated standard deviations must be due to an excess of cultures with large numbers of resistant bacteria.

Summing up the evidence, we may say that the experiments show clearly that the resistant bacteria appear in similar cultures not as random samples but in groups of varying sizes, indicating a correlating cause for such grouping, and that the assumption of genetic relatedness of the bacteria of such groups offers the simplest explanation for them.

Mutation Rate

As pointed out in the theoretical part of this paper, mutation rates may be estimated from the experiments by two essentially different methods. The first method makes use of the fact that the number of mutations in a series of similar cultures should be distributed in accordance with Poisson's law; the average number of mutations per culture is calculated from the proportion of cultures containing no resistant bacteria at the moment of the test, according to equation (5).

There are two technical difficulties involved in the application of this method. In the first place, rather large numbers of cultures have to be handled and conditions have to be chosen so that the proportion of resistant bacteria is neither too small nor too large. In the second place, the entire cultures have to be tested, which means, in our method of testing, that cultures of rather small volume have to be used and great care must be taken to plate as nearly as possible the entire culture.

Experiment No. 23 (see table 3) permits an estimate of the mutation rate by this method. Out of 87 cultures, no resistant bacteria were found in 29 cultures, a proportion of .33. From equation (5) we calculate therefore that the average number of mutations per culture in this experiment was 1.10. Since the total number of bacteria per culture was 2.4×10^8, we obtain as the mutation rate, from equation (4),

$$a = .47 \times 10^{-8} \text{ mutations per bacterium per time unit}$$

$$= .32 \times 10^{-8} \text{ mutations per bacterium per division cycle.}$$

This calculation makes use exclusively of the proportion of cultures containing no resistant bacteria. It is therefore inefficient in its use of the information gathered in the experiment.

The second method makes use of the average number of resistant bacteria per culture. The relation of this average number with the mutation rate was discussed in the theoretical part of this paper and was found to be expressed by equation (8). The mutation rates calculated by this method for each experiment are collected in table 4.

TABLE 4

Values of mutation rate from different experiments.

EXPERIMENT NO.	NUMBER OF CULTURES	VOLUME OF CULTURES	MUTATION RATE
		cc	*Mutations per bacterium per time unit*
1	9	10.0	1.8×10^{-8}
10	8	10.0	1.4×10^{-8}
11	10	10.0	4.1×10^{-8}
15	10	10.0	2.1×10^{-8}
16	20	.2*	1.1×10^{-8}
17	12	.2*	3.0×10^{-8}
21a	19	.2	3.3×10^{-8}
21b	5	10.0	3.0×10^{-8}
22	100	.2*	2.3×10^{-8}
23	87	.2*	2.4×10^{-8}
Average			2.45×10^{-8}

* Cultures in synthetic medium.

It will be seen that the values of the mutation rate obtained by the second method are all higher than the value found by the first method. This discrepancy may be traced back to the same cause as the discrepancy between the calculated and observed values of the standard deviation of the numbers of resistant bacteria. This, we found, was due to an excess of early mutations, giving rise to big clones of resistant bacteria. These big clones do not affect the mutation rate calculated by the first method, but they do affect the results of the second method, which is based on the average number of resistant bacteria.

One sees in table 4 that the mutation rate calculated by the second method does not vary greatly from experiment to experiment. In particular, it will be noted that there is no significant difference between the values obtained from cultures in broth and from cultures in synthetic medium, notwithstanding the considerable difference of metabolic activity and of growth rate of the bacteria in these two media. This shows that the simple assumption of a fixed small chance of mutation per physiological time unit is vindicated by the results. It may also be noted in table 4 that there is no significant difference between the mutation rates obtained from 10 cc cultures and those obtained from .2 cc cultures, or between the experiments with many and those with few cultures. The variability of the value of the mutation rate seems to be solely due to the peculiar probability distribution of the number of resistant bacteria in series of similar cultures predicted by the mutation theory.

At this point an experiment may be mentioned by which it was desired to find out whether or not mutations occur in a culture after the bacteria have ceased growing. A culture was grown to saturation and was then tested repeatedly for resistant bacteria and for total number of bacteria over several

days. The proportion of resistant bacteria did not change, even when the sensitive bacteria began to die, showing that the resistant bacteria have the same death rate in aging cultures as the sensitive bacteria.

DISCUSSION

We consider the above results as proof that in our case the resistance to virus is due to a heritable change of the bacterial cell which occurs independently of the action of the virus. It remains to be seen whether or not this is the general rule. There is reason to suspect that the mechanism is more complex in cases where the resistant culture develops only several days after lysis of the sensitive bacteria.

The proportion of mutant organisms in a culture and the mutation rate are far smaller in our case than in other studied cases of heritable bacterial variation. The possibility of investigation of such rare mutations is in our case merely the result of the method of detecting the mutant organisms. In other cases, the variants are detected by changes in the colony type which is produced by the mutant organism, either in the pigmentation or in the character of the surface or the edge of the colony. Often, colonies of intermediate character occur, and it is difficult to decide whether they are mixed colonies or stem from bacteria with intermediate character. This is particularly true of cases where the mutation rate is high and where reverse mutation occurs. Fairly high mutation rates, however, are a prerequisite of any study of colony variants, since the number of colonies that can be examined is limited by practical reasons.

The study of mutations causing virus resistance is free of these difficulties. The segregation of the mutant from the normal organisms occurs in the one-cell stage by elimination of the normal individuals, and the character of the colony which develops from a mutant organism is of secondary importance. Owing to the total elimination of the normal individuals, the number of organisms which may be examined is very much higher than for any other method; more than 10^8 bacteria may be tested on a single plate. Since the mutations to virus resistance are often associated with other significant characters, the method may well assume importance with regard to the general problems of bacterial variation.

It must not be supposed that the peculiar statistical difficulties encountered in our case are restricted to cases of very low mutation rates. The essential condition for the occurrence of the peculiar distribution studied in the theoretical part of this paper is the following: *the initial number of bacteria in a culture must be so small that the number of mutations which occur during the first division cycle of the bacteria is a small number*. This will always be true, however great the mutation rate, if one studies cultures containing initially a small number of organisms.

In a series of very interesting studies of the color variants of *Serratia marcescens*, BUNTING (1940a, 1940b, 1942; BUNTING and INGRAHAM 1942) succeeded to some extent in obviating the statistical difficulties by always using

inocula of about 100,000 bacteria. In some of her cases this number was sufficiently high to result in numerous mutations during the first division cycle of the bacteria. In other cases the number was apparently not high enough; since the author reports troublesome variations of the fractions of variants in successive subcultures. In those cases where the size of the inocula was high enough, the author succeeded in deriving reproducible values for the mutation rates from the study of single cultures, followed through numerous subcultures. In these cases it is sufficient to apply the equations of the theory referring to the *average* numbers of mutants as a function of time. It is clear, however, that this method is applicable only in cases of mutation rates of at least 10^{-4} per bacterium per division cycle.

In our case, as in many others, the virus resistant variants do not exhibit any striking correlated physiological changes. There is therefore little opportunity for an inquiry into the nature of the physiological changes responsible for the resistance to virus. Since the offspring of the mutant bacteria, when isolated after the test, are unable to synthesize the surface elements to which the virus is specifically adsorbed in the sensitive strain, one might suppose that this loss is a direct effect of the mutation. However, it is also conceivable that the loss occurs upon contact with virus, since it is detected only after such contact (hypothesis b_1). In some of the cases studied by BURNET (1929), where the mutational change to resistance is correlated with a change of phase, from smooth to rough or vice versa, the change of the surface structure must be a direct result of the mutation, since the mutant colonies may be picked up prior to the resistance test and, when tested, exhibit the typical change of affinity of the surface structure. These findings make it more probable that the loss of surface affinity to virus is a direct effect of the mutation.

The alteration of specific surface structures due to genetic change is a phenomenon of the widest occurrence. The genetic factors determining the antigenic properties of erythrocytes are well known. There is evidence (WEBSTER 1937; HOLMES 1938; STEVENSON, SCHULTZ, and CLARK 1939) that resistance or sensitivity to virus in plants and animals is correlated with, or even dependent on, genetic changes, possibly affecting the antigenic make-up of the cellular surface. The proof that resistance to a bacterial virus may be traced to a specific genetic change may assume importance, therefore, with regard to the general problems of virus sensitivity and virus resistance.

SUMMARY

The distribution of the numbers of virus resistant bacteria in series of similar cultures of a virus-sensitive strain has been analyzed theoretically on the basis of two current hypotheses concerning the origin of the resistant bacteria.

The distribution has been studied experimentally and has been found to conform with the conclusions drawn from the hypothesis that the resistant bacteria arise by mutations of sensitive cells independently of the action of virus.

The mutation rate has been determined experimentally.

LITERATURE CITED

BUNTING, M. I., 1940a A description of some color variants produced by *Serratia marcescens*, strain 274. J. Bact. **40**: 57–68.

1940b The production of stable populations of color variants of *Serratia marcescens* #274 in rapidly growing cultures. J. Bact. **40**: 69–81.

1942 Factors affecting the distribution of color variants in aging broth cultures of *Serratia marcescens* #274. J. Bact. **43**: 593–606.

BUNTING, M. I., and L. J. INGRAHAM, 1942 The distribution of color variants in aging broth cultures of *Serratia marcescens* #274. J. Bact. **43**: 585–591.

BURNET, F. M., 1929 Smooth-rough variation in bacteria in its relation to bacteriophage. J. Path. Bact. **32**: 15–42.

DELBRÜCK, M., and S. E. LURIA, 1942 Interference between bacterial viruses. I. Arch. Biochem. **1**: 111–141.

GRATIA, A., 1921 Studies on the d'Herelle phenomenon. J. Exp. Med. **34**: 115–131.

D'HERELLE, F., 1926 The Bacteriophage and Its Behavior. Baltimore: Williams and Wilkins.

HOLMES, F. O., 1938 Inheritance of resistance to tobacco-mosaic disease in tobacco. Phytopathology **28**: 553–561.

STEVENSON, F. J., E. S. SCHULTZ, and C. F. CLARK, 1939 Inheritance of immunity from virus X (latent mosaic) in the potato. Phytopathology **29**: 362–365.

WEBSTER, L. T., 1937 Inheritance of resistance of mice to enteric bacterial and neurotropic virus infections. J. Exp. Med. **65**: 261–286.