EXPERIMENTS WITH THE CHEMOSTAT ON SPONTANEOUS MUTATIONS OF BACTERIA

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Introduction.—All bacteria require for growth the presence of certain inorganic chemical components in the nutrient, such as potassium, phosphorus, sulphur, etc., and with a few exceptions all bacteria require an energy-yielding carbon source, such as, for instance, glucose or lactate, etc. In addition to these elements or simple compounds, certain bacteria require more complex compounds, for instance an amino acid, which they are not capable of synthesizing. For the purposes of this presentation, any of the chemical compounds which a given strain of bacteria requires for its growth will be called a "growth factor."

In general, the growth rate of a bacterial strain may be within very wide limits independent of the concentration of a given growth factor; but since at zero concentration the growth rate is zero, there must of necessity exist, at sufficiently low concentrations of the growth factor, a region in which the growth rate falls with falling concentration of the growth factor. It therefore should be possible to maintain a bacterial population over an indefinite period of time growing at a rate considerably lower than normal simply by maintaining the concentration of one growth factor—the controlling growth factor—at a sufficiently low value, while the concentrations of all other growth factors may at the same time be maintained at high values.

We shall describe further below a device for maintaining in this manner, over a long period of time, a bacterial population in the growth phase at a reduced growth rate and shall refer to it as the Chemostat.

If the growth rate of a bacterial population is reduced, it is not *a priori* clear whether the growth rate of the individual cells which constitute the population is uniformly reduced or whether a fraction of the total cell population has ceased to grow and is in a sort of lag phase, while the rest keeps growing at an undiminished rate. We believe that under the conditions of our experiments, to be described below, we are dealing with the slowing of the growth rate of the individual cells rather than the cessation of growth of a fraction of the population.

By using an amino acid as the controlling growth factor we were able to force protein synthesis in the bacterial population to proceed at a rate ten times slower than at high concentrations of that amino acid. It appears that we are dealing here with a hitherto unexplored "state" of a bacterial population—a state of reduced growth rate under the control of a suitably chosen growth factor.

The study of this "slow-growth-phase" in the Chemostat promises to yield information of some value on metabolism, regulatory processes, adaptations and mutations of micro-organisms; the present paper, however, is concerned only with the study of spontaneous mutations in bacteria.

There is a well-known mutant of the B strain of coli, B/1, which is resistant to the bacterial virus T_1, sensitive to the bacterial virus T_5, and which requires tryptophane as a growth factor. We used this strain and mutants derived from it in all of our experiments here reported. As a nutrient medium we used a simple synthetic lactate medium (Friedlein medium) with tryptophane added. As the controlling growth factor, we used either lactate or tryptophane.

Experiments on Growth Rates at Low Tryptophane Concentrations.—In order to determine the growth rate of B/1 as a function of the tryptophane concentration (at high lactate concentrations) we made a series of experiments in which we incubated at 37° at different initial tryptophane concentrations c, flasks inoculated with about 100 bacteria per cc. and obtained growth curves by determining (by means of colony counts) the number of viable bacteria as a function of time. Because the bacteria take up tryptophane, the tryptophane concentration c decreases during the growth of the culture and the growth rate for the initial tryptophane concentration c must therefore be taken from the early part of the growth curve.

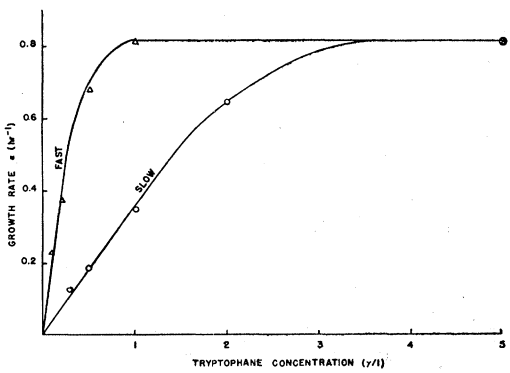

FIGURE 1

Experiment of September 18, 1950, at 37°C. The curve marked SLOW relates to strain B/1 and the curve marked FAST relates to B/1/f.

The growth rate α is defined by

$$\alpha = \frac{1}{n}\frac{dn}{dt}$$

where n is the number of bacteria per cc. The reciprocal value, $\tau = \frac{1}{\alpha}$, we shall designate as the "generation time." From the generation time thus defined, we obtain the time between two successive cell divisions by multiplying by ln 2.

In figure 1 the curve marked "slow" shows the growth rate α as a function

of the tryptophane concentration c for 37°. At low tryptophane concentrations c, the growth rate at first rises proportionately with the concentration; with increasing concentrations, however, the growth rate approaches a limit and for concentrations above 10 γ/l. (micrograms per liter) the growth rate is no longer appreciably different from its highest attainable value. This highest value corresponds to a generation time of $\tau = 70$ min. One half of the highest value is reached at a tryptophane concentration of about $c = 1$ γ/l. This concentration corresponds to about three molecules of tryptophane per 10^{-12} cc. (The volume of one bacterium is about 10^{-12} cc.)

The proportionality of the growth rate with the concentration of tryptophane at low concentrations becomes understandable if we assume that the uptake and utilization of tryptophane by the bacterium requires that a tryptophane molecule interact with a molecule of a certain enzyme contained in the bacterium and that the uptake of tryptophane by these enzyme molecules in the bacterium becomes the rate-limiting factor for the growth of the bacterium. On the basis of this argument, we believe that down to as low concentrations of tryptophane as the proportionality of growth rate to concentration can be experimentally demonstrated, the observed growth rate of the bacterial culture represents the growth rate of the individual bacterium and that no appreciable fraction of the population goes into lag.

The Theory of the Chemostat.—In the Chemostat, we have a vessel (which we shall call the growth tube) containing V cc. of a suspension of bacteria. A steady stream of the nutrient liquid flows from a storage tank at the rate of w cc./sec. into this growth tube. The content of the growth tube is stirred by bubbling air through it, and the bacteria are kept homogeneously dispersed throughout the growth tube at all times. An overflow sets the level of the liquid in the growth tube, and through that overflow the bacterial suspension will leave the growth tube at the same rate at which fresh nutrient enters the growth tube from the storage tank.

After a certain time of such operation, at a fixed temperature, a stationary state is reached in the growth tube. We are interested in this stationary state in the particular case in which the growth rate of the bacteria is determined by the concentration in the growth tube of a single growth factor (in our specific case tryptophane). By this we mean that the concentration of a single growth factor (tryptophane) in the growth tube is so low that a small change in it appreciably affects the growth rate of the bacteria, and at the same time the concentration of all other growth factors in the growth tube is so high that a small change in them has no appreciable effect on the growth rate of the bacteria. As we shall show, under these conditions the concentration c of the growth factor in the growth tube *in the stationary state*, for a fixed flow rate w, will be independent of the

concentration a of this growth factor in the nutrient liquid in the storage tank.

In order to see this, we have to consider the following:

1. For zero flow rate of the nutrient ($w = 0$), the bacterial concentration n would rise in the growth tube according to $\frac{1}{n}\frac{dn}{dt} = \alpha(c)$, where α is the growth rate which, according to our premise, is a function of the concentration, c, of the growth factor.

2. In the absence of *growth*, the bacterial concentration in the growth tube would decrease for a given flow rate w according to the formula

$$\frac{1}{n}\frac{dn}{dt} = \frac{-w}{V}$$

where $\frac{w}{V} = \beta$ may be called the "washing-out rate" of the growth tube, and $\frac{1}{\beta}$ the washing-out time.

After a while, for any given flow rate w, a stationary state will be reached in the Chemostat at which the growth rate α will be equal to the washing-out rate β (and the generation time τ equal to the washing-out time $\frac{1}{\beta}$), i.e.,

$$\alpha(c) = \beta = \frac{w}{V}; \qquad \tau = \frac{1}{\beta} = \frac{V}{w}. \tag{1}$$

Thus, in the stationary state for any fixed flow rate w, the growth rate α is fixed; since α is a function of the concentration c in the growth tube, it follows that c is also fixed and independent of the concentration a of the growth factor in the storage tank.

It may be asked what is the mechanism by which, for different values of a but the same flow rate w, the same concentration c establishes itself in the growth tube in the stationary state. Clearly what happens is this: Suppose that, for a certain concentration a_1 of the growth factor in the storage tank, a stationary state with the concentration c in the growth tube has established itself and subsequently the concentration of the growth factor in the storage tank is suddenly raised to a higher value a_2. When this change is made, the concentration c in the growth tube will at first rise and along with it will rise α, the growth rate of the bacteria, which is a function of c. The concentration of the bacteria in the growth tube will thus start to increase, and therefore the bacteria will take up the growth factor in the growth tube at an increased rate. As the increase of the bacterial concentration continues, the growth rate of the bacteria will, after a while, begin to fall and will continue to fall until a new stationary state is reached

at which the bacteria again grow at the same rate at which they are washed out, i.e., for which again we have $\alpha = \frac{w}{V}$. When this state is reached, the concentration of the growth factor in the growth tube is again down to the same value c which it had before the concentration of the growth factor in the storage tank was raised from a_1 to a_2, while the bacterial density is now higher.

In the stationary state the tryptophane balance requires that the following equation hold:

$$a = c + n \frac{V}{w} F(c) \tag{2}$$

or

$$a = c + n \frac{F(c)}{\alpha(c)} \tag{3}$$

where $F(c)$ gives in grams per second the amount of the growth factor which one bacterium takes up per second.

As can be easily seen, the amount Q of the growth factor that is taken up per bacterium produced is given by

$$Q = \frac{F(c)}{\alpha(c)}$$

so that, for the stationary state, we may also write

$$a = c + nQ \quad \text{or} \quad n = \frac{a - c}{Q} \tag{4}$$

and for the $c \ll a$ we may write

$$n = \frac{a}{Q}. \tag{5}$$

The Use of Tryptophane as the Controlling Growth Factor.—Since in the stationary state the tryptophane concentration in the growth tube of the Chemostat is always below 10 γ/l. whenever the generation time is appreciably above 70 min., we may use the approximation given in equation (5) whenever the tryptophane concentration a in the storage tank is above 100 γ/l.

In order to determine the amount of tryptophane, Q, taken up per bacterium produced, we grew bacterial cultures in lactate medium with varied amounts of tryptophane added. We found that if the initial tryptophane concentration is kept below 10 γ/l., then the amount of tryptophane taken up per bacterium produced is not dependent on the tryptophane concentration and has a value of $Q = 2 \times 10^{-15}$ gm. At higher tryptophane concentrations, however, more tryptophane is used up per bacterium produced.

From equation (5), using the value of $Q = 2 \times 10^{-15}$ gm. we obtain $n = 5 \times 10^7$/cc. for $a = 100$ γ/l. and we obtain $n = 5 \times 10^8$/cc. for $a = 1000$ γ/l.

From this, it may be seen that, by choosing suitable values for a and w, we may vary over a wide range, independently of each other, the bacterial concentration n and the tryptophane concentration c.

When we grew B/1 in a Chemostat ($V = 20$ cc.) for ten days at 37° at a generation time of $\tau = 2$ hrs. and at a bacterial density of 5×10^8/cc., we found that a change from the original bacterial strain, B/1, had taken place. The new strain, which we shall designate as B/1/f, differs from the original strain only inasmuch as it grows, at very low tryptophane concentrations, about five times as fast as the original strain. The growth rate at higher tryptophane concentrations is not perceptibly different, nor could we detect any other difference between the two strains. The curve marked "fast" in figure 1 gives the growth rate of the B/1/f strain as a function of the tryptophane concentration at 37°.

The ability of the B/1/f strain to grow faster at very low tryptophane concentrations gives it an advantage over the B/1 strain under the conditions prevailing in the growth tube of the Chemostat; and a mutant of this sort must, in time, displace the original strain of B/1.

Because in our experiments we would want to avoid—as much as possible—population changes of this type in the Chemostat, we used in all of our experiments reported below this new strain, B/1/f.

Spontaneous Mutations in the Chemostat.—If we keep a strain of bacteria growing in the Chemostat and through spontaneous mutations another bacterial strain is generated from it, then the bacterial density n^* of the mutant strain should (for $n^* \ll n$) increase linearly with time, provided that, under the conditions prevailing in the Chemostat, the new strain has the same growth rate as the original strain, so that there is no selection either for or against the mutant. In the absence of selection we have

$$\frac{dn^*}{dt} = \frac{\lambda}{\tau} n \tag{6}$$

where n^* is the density of the mutant population, n is the density of the population of the parent strain and λ the number of mutations produced per generation per bacterium. Equation (6) holds under the assumption that back mutations can be neglected. From (6), we obtain for $n^* \ll n$

$$\frac{n^*}{n} = \frac{\lambda}{\tau} t + \text{Const.} \tag{7}$$

From this it may be seen that—as stated above—the relative abundance of the mutants must increase linearly with time if there is no selection for or against the mutant.

If the growth rate of the mutant strain is smaller than the growth rate of the parent strain ($\alpha^* < \alpha$) so that there is selection against the mutant in the growth tube of the Chemostat, then the density n^* of the mutant population should—after an initial rise—remain constant at the level given by

$$\frac{n^*}{n} = \frac{\alpha}{\alpha - \alpha^*} \lambda. \qquad (8)$$

Experiments on Spontaneous Mutations in the Chemostat.—Of the various mutations occurring in a growing bacterial population, mutants resistant

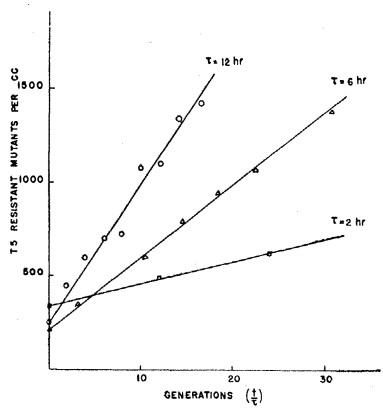

FIGURE 2

Experiments of May 3, 8, and 28, 1950, at 37°C. giving for strain B/1/f for three different values of the generation time the concentration of the mutants resistant to T_5, for a population density of 5×10^8 bacteria per cc.

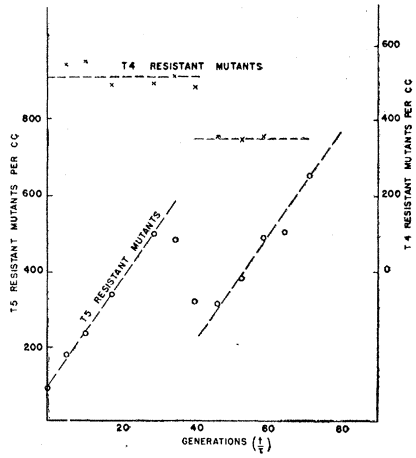

FIGURE 3

Experiment of July 19, 1950, at 37°C. giving for strain B/1/f the concentration of mutants resistant to T_5 (left-hand scale) and mutants resistant to T_4 (right-hand scale) for a population density of 2.5×10^8 bacteria per cc. In this experiment oxygen containing 0.25% CO_2 was used for aeration.

to a bacterial virus are perhaps the most easily scored with considerable accuracy. In our experiments we mostly worked with mutants of our coli strain which were resistant to the bacterial viruses T_5 or T_6.

When we grow the strain B/1/f in the Chemostat with a high concentration of tryptophane but a low concentration of lactate in the nutrient in the storage tank, so that lactate rather than tryptophane is the controlling growth factor, we find—after a short initial period—that the bacterial densities of the mutants resistant to T_5 or T_6 each remain at a constant level. These levels appear to correspond to a selection factor

$\frac{\alpha - \alpha^*}{\alpha}$ of a few per cent.

We are inclined tentatively to assume that the behavior of these two mutants exemplifies the general rule that the vast majority of all the different mutational steps leading away from the wild type yield mutants which—under conditions of starvation for the carbon source—grow slower than the parent type.

On the other hand, if we grow our tryptophane-requiring strain in the Chemostat with a high concentration of lactate but a low concentration of tryptophane in the nutrient in the storage tank (so that tryptophane rather than lactate is the controlling growth factor) and if we run the Chemostat at a generation time well above 70 min. (the generation time at high tryptophane concentrations)—then there is no reason to expect mutants *in general* to grow appreciably slower than the parent strain, particularly if the growth of the parent strain is kept very slow by keeping the tryptophane concentration in the growth tube very low. In this case one would rather expect a mutation to affect the growth rate only if it affects the uptake or utilization of tryptophane by the bacterium or if the mutant is a very slow grower. Accordingly, we should, in general, expect the mutant population to increase linearly with time in the Chemostat when tryptophane is used as the controlling growth factor.

Figure 2 gives for 37° the experimental values for the bacterial density for the mutant population resistant to T_5 in the growth tube of the Chemostat as a function of the number of generations through which the parent strain has passed in the Chemostat. $\left(\text{Number of generations } g = \frac{t}{\tau}.\right)$ The three curves in the figure correspond to generation times of 2 hours, 6 hours and 12 hours. The slope of the straight lines gives λ, the mutation rate per generation, as 2.5×10^{-8}; 7.5×10^{-8}; and 15×10^{-8} per bacterium. We see that the mutation rate per generation for $\tau = 6$ hours is three times as high and for $\tau = 12$ hours is six times as high as it is for $\tau = 2$ hours. Thus the mutation rate per generation is, in our experiment, not constant but increases proportionately with τ and what remains constant is the number of mutations produced per unit time per bacterium. According to the above figures, we have $\frac{\lambda}{\tau} = 1.25 \times 10^{-8}$ per hour per bacterium.

This result is not one that could have been foreseen. If mutants arose, for instance, as the result of some error in the process of gene duplication, then one would hardly expect the probability of a mutation occurring per cell division to be inversely proportionate to the rate of growth.

If the processes of mutation could be considered as a monomolecular reaction—as had been once suggested by Delbruck and Timofeeff-Ressovsky—then, of course, the rate of mutation per unit time should be

constant. The rate k of a monomolecular reaction is given by

$$k = Ae^{-W/RT}. \quad (9)$$

The value of the constant A can be calculated from the observed reaction rate k and the heat of activation W (which can be obtained by determining the temperature coefficient of the reaction).

Using the Chemostat, we have determined the rate of mutation to resistance to T_5 at 25° (for $\tau = 6$ hrs. and $\tau = 12$ hrs.) and found it to be about one half of the mutation rate at 37°. From this value and the mutation rate of $\frac{\lambda}{\tau} = 1.25 \times 10^{-8}$ per hour per bacterium at 37° we compute $A \approx 10^{-3}$ per sec.

In a condensed system, such as an aqueous solution, A has been found to lie between 10^5 and 10^{14} per sec. for known monomolecular reactions. Therefore if the mutation studied by us were due to a monomolecular reaction, it would have an A value 10^8 times lower than the lowest value so far found.

The density of the mutants resistant to the bacterial virus T_6 in the Chemostat, with tryptophane as the controlling growth factor, also appears to rise linearly with time for $\tau = 2$ hours, $\tau = 6$ hours and $\tau = 12$ hours, but our results so far are not sufficiently accurate to say whether this mutation also occurs at a constant rate per unit time for different generation times τ. The temperature coefficient of the mutation rate appears to be very low, but again this conclusion must await more accurate experiments.

The result obtained for mutation to resistance to the virus T_5, showing that this mutation occurs at a constant rate per unit time up to a generation time of $\tau = 12$ hours, raises the question whether this is generally true of spontaneous bacterial mutations or whether we are dealing in our case with certain exceptional circumstances. Clearly, a number of different mutations will have to be examined, different amino acids will have to be used as the controlling growth factor and other conditions will have to be varied before one would draw the far-reaching conclusion that our observation on mutation to resistance to the virus T_5 exemplifies a general rule.

Mutants Resistant to T_4.—We find that mutants resistant to T_4 are selected against in the Chemostat when grown either with lactate or with tryptophane as the controlling growth factor, i.e., the number of mutants remains—after an initial rise—at a fixed level.

It is known that of the different mutants of the B strain of coli which are resistant to the virus T_4, the most frequent one is also resistant to the viruses T_3 and T_7 and that this mutant is a very slow grower under ordinary conditions of culture. It is conceivable that this might explain why the mutants resistant to T_4 are selected against in the Chemostat even when

the bacterial population grows under tryptophane control and at a much reduced rate.

Manifestation of "Evolution" in the Chemostat.—If a bacterial strain is grown over a long period of time in the Chemostat, from time to time a mutant might arise which grows faster, under the conditions prevailing in the Chemostat, than the parent strain. If this happens, practically the entire bacterial population in the Chemostat will change over from the parent strain to the new strain. We have discussed one change-over of this sort, i.e., the change-over from the strain B/1 to the strain B/1/f. There is no reason to believe, however, that no further change-over may take place when we start out with B/1/f as the parent strain and continue to grow it in the Chemostat over a long period of time.

We have seen that the mutants resistant to T_5 accumulate in the Chemostat and that their number rises linearly with the number of generations, giving a straight line, the slope of which is given by λ. If now at a certain time the population changes over in the Chemostat from the parent strain to a faster-growing strain, the accumulated mutants resistant to the bacterial virus T_5 which were derived from the parent strain should disappear from the Chemostat along with the parent strain. This should lead to a fall in the number of mutants resistant to the bacterial virus T_5 during a change-over from the parent strain to the faster-growing strain. After the change-over to the new strain, the concentration of the mutants resistant to T_5 may be expected again to increase linearly with the number of generations, giving a straight line which has the same slope as before the change-over, because the new strain which displaces the parent strain may be expected to mutate to resistance to T_5 at an unchanged rate λ.

Thus, we may in general expect, when a change-over in the population takes place, the concentration of the mutants resistant to T_5 to shift from one straight line which lies higher to another, which lies lower. The magnitude of this shift may be somewhat different from experiment to experiment, depending on when mutants resistant to T_5 happen to make their first appearance in the population of the new strain.

At the outset, the bacteria belonging to the new strain will be few in number but their number will increase exponentially with the number of generations until—at the time of the change-over—the bacteria belonging to the new strain become an appreciable fraction of the total population. If the mutation rate to resistance to T_5 is of the order of magnitude of 10^{-8}, then it is unlikely that such a mutant should appear in the population of the new strain until its population has reached perhaps 10^7. However, because an element of chance is involved, occasionally a mutant resistant to T_5 may appear earlier and, if that happens, the "shift" associated with the change-over will be smaller and in principle it might even be negative.

If a bacterial population remains growing in the Chemostat for a sufficiently long time, a number of such change-overs might take place. Each

such step in the evolution of the bacterial strain in the Chemostat may be expected to manifest itself in a shift in the ascending straight line curve of the T_5 resistant mutants.

As we have seen, the mutants resistant to T_4 remain—apart from an initial rise—at a constant level in the Chemostat. However, when the bacterial population in the Chemostat changes over from a parent strain to a new strain, the T_4 resistant mutants might change over from one level to another, because the selection against the two strains might be different.

Figure 3 shows, for mutants resistant to T_5 and for mutants resistant to T_4, the number of mutants as a function of the number of generations $\frac{t}{\tau}$ in a Chemostat which was run for 300 hours at $\tau = 4$ hours with tryptophane as the controlling growth factor.

It may be seen that these two curves show a population change-over of the type just described. The curve for the T_5 resistant mutants shows a shift, P, of $P = 32$ generations.

A number of shifts of this type were observed in different experiments. We verified that these "shifts" represent population change-overs by showing in one case that (under the conditions prevailing in the chemostat) bacteria taken from the Chemostat before the change-over in fact grow slower than bacteria taken from the Chemostat after the change-over.

In order to show this, we took from the Chemostat before the change-over a bacterium resistant to T_5 and after the change-over a bacterium sensitive to T_5 and inoculated a *second* Chemostat (operated under identical conditions) with a 50-50 mixture of these two strains. We then found that the relative abundance of the resistant strain rapidly diminished. In the corresponding control experiment we took a sensitive bacterium from the Chemostat before the population change-over and a resistant one after the population change-over and again found that the strain prevalent before the change-over (this time the sensitive one) was the slower grower.

In the later stages of the change-over the concentration x of the original strain falls off exponentially with the number of generations, $g = \frac{t}{\tau}$, so that we may write $x = Ce^{-g/\gamma}$. In our experiment we obtained for γ a value of $\gamma = 3.25$.

It should be noted that the value of γ can be read also directly (though not accurately) from the curve, which gives the concentration n^* of the resistant mutants as the function of g, the number of generations. During the change-over the concentration c of the tryptophane in the growth tube goes over from an initial value c_1 to a final, lower value c_2 and it can be shown that for the midpoint of the change-over at which $c = \frac{c_1 + c_2}{2}$ we have

$$\gamma = \frac{P/4}{1 - \frac{1}{\lambda n}\frac{dn^*}{dg}} + \frac{1}{2} \qquad (10)$$

where P is the magnitude of the shift expressed in the number of generations by which the ascending straight line of the resistant mutants is shifted in the change-over. This formula holds only if τ is large so that the rate of growth of the bacteria in the Chemostat is proportionate to the tryptophane concentration c. Because the exact position on the curve of the midpoint of the change-over on the curve n^* is not known, this formula can give only a rough indication for the value of γ.

In our case, the estimate based on it gave for γ a value of $\gamma = 2.4$ in place of the directly observed value of $\gamma = 3.25$. Within the limits of the accuracy of our curve for n^* these two values are consistent with each other.

Population change-overs manifesting themselves in a shift in the ascending straight line of the T_5 resistant mutants occurred in every experiment carried at $\tau = 4$ hrs. beyond the 50th generation. In an experiment carried to the 450th generation at a bacterial density of 2.5×10^8/cc., a number of such shifts occurred, the last one at about the 350th generation. (In the course of this experiment the mutants resistant to T_4 rose twice from a low level to a high peak, the first of which reached 4.6×10^4 and the second 4.5×10^6 mutants per cc. This phenomenon is now being investigated.)

It may be said that our strain, if grown in the Chemostat at low tryptophane concentration for a long period of time, undergoes a number of mutational steps, each one leading to a strain more "fit" than the previous one, and that each step in this process of evolution becomes manifest through the shifts appearing in the curve of the mutants resistant to T_5.

THE ANALYSIS OF A CASE OF CROSS-STERILITY IN MAIZE

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The male gametophyte of maize is extremely sensitive to chromosomal unbalance. Duplications and deficiencies are almost always accompanied by reduced pollen transmission. The functioning of the male gametophyte is also known to be affected by a number of genic factors. The most thoroughly studied locus and probably the most interesting is Ga_1 on chromosome 4. First detected by Correns[3] because of aberrant F_2 ratios for sugary-starchy, this factor was subsequently studied by Jones,[8, 10]