Apr 20/50

Growth Factor Limited Bacterial Populations Kept in the Growth Phase

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All bacterial strains require for growth the presence of certain chemical components in the nutrient liquid, such as potassium, phosphorus, sulphur, etc., and with a few exceptions all bacterial strains require an energy yielding carbon source, such as for instance glucose or lactate, etc. In addition to these elements or simple compounds, certain bacterial strains may require more complicated 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 the following, by way of illustrating a general principle, we shall use as an example a mutant of the B strain of coli which -- in addition to the compounds required by the B strain itself -- requires one growth factor, tryptophane, and we shall single out this one factor which is necessary for its growth for special attention.

In general, the growth rate of a bacterial strain may be independent, within very wide limits of concentrations, of the concentration of a given growth factor. Since, however, at zero concentrations of the growth factor 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 growth factor.

In our particular case, with tryptophane as a growth factor, it turned out that the growth rate drops to about half of its value at

high concentrations if the concentration is lowered to $1.5\ 10^{-9}$ gm/cc or 1.5 γ/liter. At this very low concentration, we have about five molecules of tryptophane per 10^{-12}cc of the nutrient (10^{-12}cc being the volume of one bacterium).

The growth rate α is defined by

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

where n is the number of bacteria per cc.

In our case, α is a function of the tryptophane concentration c and α(c) rises at very low concentrations with increasing concentration c, practically reaches saturation, i.e. $\alpha \to \infty$ which we may call the "normal" growth rate, at concentrations of the order of magnitude of 10 γ/liter of tryptophane and then remains constant.

In the following, we shall describe an arrangement in which bacteria can be maintained in the growth phase, over very long periods of time, at such low tryptophane concentrations at which the growth rate is appreciably below normal. We shall refer to our apparatus in which bacteria are grown under such conditions as a "Chemostat."

Let us consider the following system: a vessel (which we shall call hereafter the growth tube) containing V cc of nutrient is inoculated with a strain of bacteria. A steady stream of the nutrient flows from a storage tank at the rate of w cc/sec into this growth tube. The content of the growth tube is stirred, for instance 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 under the particular condition in which the growth rate of the bacteria in the growth tube is determined by the concentration of a single growth factor. By this we mean that the concentration of a single growth factor is so low that a change in it appreciably affects the growth rate of the bacteria, and at the same time, the concentration of all other growth factors is so high that a change in them has no appreciable effect on the growth rate of the bacteria. Under these conditions, the concentration c of the growth factor in the growth tube in the stationary state will, for a fixed flow rate w, 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 $\frac{w}{V}$ may be called the "washing-out rate" of the growth tube.

For any given flow rate w, after a while a stationary state will be reached in the chemostat at which the growth rate will be equal

to the washing-out rate; i.e.

$$(1) \qquad \alpha(c) = \frac{w}{V}$$

Thus in the stationary state, for any fixed flow rate w, the growth rate is fixed; therefore the concentration c in the growth tube 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 a stationary state c has established itself for a certain concentration a_1 of the growth factor in the storage tank and the concentration of the growth factor in the storage tank is suddenly raised to a higher value a_2; then the concentration c in the growth tube will at first also 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 increase, and therefore the bacteria will take up the growth factor from 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 it 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. When that 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.

As can be easily seen in the stationary state, the following equation must hold:

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

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

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

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

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

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

$$(4) \qquad a = c + nA$$

In the case of our tryptophane requiring strain, we find that if the 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 $2\ 10^{-15}$ gm. (At higher concentrations of tryptophane more tryptophane is taken up per bacterium produced.)

Whenever the washing-out rate is appreciably below the "normal" growth rate α_∞, the tryptophane concentration c in the growth tube is below 10 γ/l. For tryptophane concentrations a in the storage tank which are larger by a factor of 10 or more than 10 γ/l, we may therefore write with good approximation from (4)

$$(5) \qquad n \approx \frac{a}{A}$$

For a value of a = 1000 γ/l, we have for instance, a value of n = 5×10^8/cc, and for a value of a = 100 γ/l we have a value of n = 5×10^7. From this it may be seen that we can vary, over a wide range, independently the bacterial concentration n and the tryptophane concentration c by choosing the proper value of a and w.

Description of Apparatus

A tube leading to the bottom of the storage tank (see Figure) is connected to a small air compressor (for instance a little air pump such as used for aereating aquaria). When the compressor is first started, the air rises rapidly in bubbles through the nutrient liquid and accumulates in the space above the liquid level until the pressure in the nutrient liquid at the bottom of the storage tank becomes equal to the air pressure in the tube. The air space in the storage tank above the liquid level communicates through a narrow capillary with the outside air and therefore the air will continue to bubble through the nutrient liquid in the storage tank even though at a very slow rate (of perhaps one bubble per minute).

The pressure of the air entering the tube which leads to the bottom of the storage tank is regulated by a simple "pressure regulator" consisting of an air outlet located at the bottom of a glass cylinder which is filled with water up to a certain level. Above this level, the air communicates freely with the outside air. By changing the water level in the "pressure regulator," the air pressure can be adjusted to any value required for the operation of the chemostat.

In this arrangement, the pressure at the bottom of the storage tank will always be greater than the pressure of the outside air by the height of the water column in the pressure regulator, and thus the pressure at the bottom of the storage tank will be independent of the height of the level of the nutrient liquid in the storage tank. This is important because the level of the nutrient liquid will gradually diminish during the operation of the chemostat.

From the storage tank, the nutrient liquid is pressed through a sintered glass filter and a capillary, into the growth tube, and is mixed drop by drop with the liquid nutrient contained in the growth tube. The growth tube is aereated, and its content is thus continuously stirred.

The level of the liquid in the growth tube is set by a syphon and is maintained constant. The nutrient liquid and the bacteria suspended in it leave the growth tube through the syphon at the same rate at which fresh nutrient enters the growth tube.

The air space above the nutrient liquid in the growth tube communicates with the outside air. Thus the pressure which forces the nutrient liquid through the sintered disk and the capillary is at all times equal to the height of the water column in the pressure regulator.

If the chemostat has been in operation for some time and then the barometric pressure falls very suddenly, the pressure of the air entering into the storage tank also falls suddenly, and the nutrient liquid will enter at the bottom of the storage tank into the air pressure tube and will rise in it up to a certain height. If this happens, then the pressure at the bottom of the storage tank no longer exceeds the outside pressure by the height of the water column in the regulator, but rather by a greater amount, and the flow of the nutrient liquid into the growth tube increases. Because of the communication between the air space above the nutrient liquid in the storage tank through a capillary with the outside air, this condition will be, however, very quickly corrected. As air flows out of the storage tank through this capillary outlet, the pressure at the bottom of the storage tank diminishes and the level of the nutrient liquid in the air pressure tube in the

storage tank falls again. Thus within a short period of time, the pressure at the bottom of the storage tank is restored to its former value.

Thus the chemostat permits to keep the flow rate of the nutrient liquid into the growth tube constant independently of changes in barometric pressure and of the changes in the liquid level in the storage tank. The flow rate can be changed as desired by changing the water level in the pressure regulator.

Determination of the Growth Rate α:

The chemostat should permit to determine the growth rate α as a function of the concentration of the growth factor, for concentrations at which the growth rate changes appreciably with changing concentration.

If the concentration a of the growth factor in the storage tank is so low as to be in the region in which the growth rate is sensitive to the concentration, the growth rate for the concentration a can be determined by determining the flow rate w_o for which, in the stationary state, the bacterial concentration n in the growth tube becomes zero. As can be seen from equation (4), if the bacterial concentration n in the growth tube becomes zero, then we have $c = a$, and since according to equation (1), we have $\alpha(c) = \frac{w}{V}$ it follows that

$$\alpha(a) = \frac{w_o}{V} \tag{6}$$

In practice, it will be easier to determine, in place of w_o, the flow rate w_n for which the bacterial concentration n in the growth tube has, in the stationary state, a low value. We may write

$$\alpha(a - nA) = \frac{w_n}{V}$$

and if $n \ll \frac{a}{A}$, then the value of $(a - nA)$, the growth factor concentration in the growth tube, calculated from n, will be only slightly affected by the inaccuracy in the determination of the value of A.

In order to obtain the growth rate as a function of the growth factor concentration, it will, however, be necessary in this instance, to determine the growth rate for a number of growth factor concentrations, a_1, a_2, a_3, etc. in the storage tank.

This need not be done if a different method is used which is as follows:

The bacterial concentration n in the growth tube is determined in the stationary state, for a number of different flow rates like w_1, w_2, w_3, etc. for each of two different concentrations of a_1 and a_2 of the growth factor in the storage tank. For any one of these flow rates, we have then two equations corresponding to equation (2).

$$(7) \qquad a_1 = c + n_1 \frac{V}{w} F(c)$$

$$a_2 = c + n_2 \frac{V}{w} F(c)$$

For any one given flow rate, the concentration c in the growth tube is the same, and so is the value of F(c). We have, therefore, two equations with two unknowns, c and F(c), for which we can solve these equations.

$$(8) \qquad F(c) = \frac{w}{V} \frac{a_1 - a_2}{n_1 - n_2}$$

$$c = \frac{a_1 n_2 - a_2 n_1}{n_1 - n_2}$$

Thus we obtain for a given flow rate w, the concentration c for the growth rate $\alpha = \frac{w}{V}$.

In the case of either of these two methods for the determination of the growth rate as a function of the concentration of the

growth factor, it is important to make certain that bacteria do not stick to the wall of the growth tube. Coating the inner surface of the growth tube with silicones and providing for vigorous stirring of the growth tube by aereation is therefore advisable in such experiments.

Since at the low concentrations of the growth factor in the storage tank which have to be used the bacterial concentration in the growth tube is very small, it is advisable to remove even traces of toxic substances from the nutrient liquid. Two methods are available for this purpose:

a) We may add .05% of sodium citrate to the nutrient medium to remove heavy metal ions.

b) We may add a small amount of the growth factor to the medium, sufficient to permit the bacteria to grow up to a concentration of 5×10^6/cc, inoculate with the growth factor requiring strain, incubate at 37° for 48 hours, pasteurize the medium and then add the desired amount of growth factor to it.

Another Method of Determining the Growth Rate:

The growth rate at very low concentrations of tryptophane can also be determined by inoculating with a small inoculum (of the order of 200 bacteria per cc) growth flasks containing different tryptophane concentrations ranging, for instance, from 0.05 γ/l to 30 γ/l, incubating these growth flasks over a long period of time and making, at regular intervals, colony counts of the bacteria in samples taken from these growth flasks.

Preliminary experiments carried out with our tryptophane requiring strain of coli, showed that the growth rate increases with

increasing tryptophane concentration and reaches saturation at higher concentrations. The growth rate was determined for low concentrations down to about 1/10 of the normal growth rate at 37° C, and up to this limit it was found, for low tryptophane concentrations, to be about proportionate to the concentration.

For a tryptophane requiring strain of coli -- the "fast" strain -- which is different from our original tryptophane requiring strain (see later), we determined at 37° C for high concentrations of tryptophane, the growth rate, and found it to be, by a factor of 1.7, higher than its growth rate at 25° C. With decreasing tryptophane concentrations, this factor decreases; and within the accuracy of our measurements, it becomes unity for a tryptophane concentration of about 0.5 γ/l when the growth rate is at about 1/2 of its normal value at 37°.

That the growth rate is independent of the temperature at low tryptophane concentrations can be understood if we assume that the limiting process for growth at such low tryptophane concentrations consists in the reaction of tryptophane with an enzyme contained in the bacterium and that the heat of activation for this reaction is small.

Adaptations and Mutations of the Bacterial Populations in the Chemostat:

A bacterial population which is kept in the growth phase in the chemostat will undergo changes due to adaptation of the whole population or to the mutation of a few individual bacteria and the chemostat may permit us to distinguish between these two types of changes by letting the bacteria grow once at a high bacterial density and once at a low bacterial density at the same concentration c of the growth factor

in the growth tube. Changes which are due to adaptation will manifest themselves independently of whether the concentration of the bacteria is high or low, but changes due to mutations will be dependent on the size of the total bacterial population and therefore will be dependent on the concentration n of the bacteria.

By growing our tryptophane requiring strain of bacteria for about eight days in the chemostat at a growth rate of about 1/2 of the normal growth rate at a bacterial concentration of $n = 5 \times 10^8$ per cc, the bacterial population underwent a change of its growth rate characteristic. The new strain when tested for its growth rate in growth flasks, turned out to grow about five times as fast at low concentrations of tryptophane as our original tryptophane requiring strain. When the experiment was repeated in another run, the new strain again appeared after about eight days of growth in the chemostat at a concentration of 5×10^8/cc. But in a third run in which the bacteria were grown for about eight days in the chemostat at the concentration of $n = 5 \times 10^7$/cc, the new strain did not appear. This suggests that we are dealing here not with an adaptation, but rather with a mutation of our original tryptophane requiring strain which at low tryptophane concentrations grows slowly -- and will be called hereafter the "slow" strain -- to a strain which at low tryptophane concentrations grows about five times as fast, and will be called hereafter the "fast" strain.

The Appearance of Mutants in the Chemostat and the Mutation Rate:

Bacteria growing in the chemostat will undergo various mutations. They will, for instance, mutate to resistance to the bacterial viruses T_4, T_5, and T_6. Assuming that the growth rate of the mutant is

lower than the growth rate of the original bacterial strain, the relative abundance of the mutant will first grow and then reach saturation.

The number of mutants will first grow according to the equation

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

where n^* is the number of mutants, λ is a constant different for each mutation, and $\frac{\lambda}{\alpha}$ is the number of mutants appearing per generation (one generation being defined by the multiplication of the number of bacteria by the factor e).

Neglecting the possibility of back mutation and assuming that the only mutation that is taking place in the chemostat goes from the original strain to the mutant strain, that we have singled out for our attention, we have for the stationary state when saturation is reached

$$\frac{n^*}{n} \quad \frac{\lambda}{\alpha} \frac{\alpha}{\alpha - \alpha^*}$$

where α^* is the growth rate of the mutant.

A preliminary experiment with the "fast" tryptophane requiring strain has shown that the relative abundance of mutants resistant to T_5 rises over a period of several days at the rate of

$$\frac{1}{\alpha} \frac{dn^*}{dt} = \frac{\lambda n}{\alpha} \approx 2 \times 10^{-8} n.$$

The mutants resistant to T_4 showed a peculiar behavior which is being further investigated. In this experiment, the chemostat was run at the rate of $\frac{V}{w}$ = 2 hours, corresponding to a growth rate of about one-half of the normal growth rate and the bacterial density in the growth tube was about 10^8/cc.

Mutation Experiments with the Chemostat:

The chemostat should make it possible to study a number of questions relating to mutations.

1) It should enable us to determine whether the mutation rate per generation depends on the growth rate when the growth rate is depressed by keeping the concentration of one growth factor low.

2) It should enable us to determine whether, if a bacterial strain requires two growth factors, and is capable of several mutations, the ratio of these mutations depends on which growth factor is made growth rate limiting.

3) It should enable us to determine whether any given chemical agent is mutagenic; i.e., increases the mutation rate.

Experiments on Bacterial Regulation with the Chemostat:

The chemostat should enable us to determine whether bacteria are capable of regulating their metabolism in order to economically adjust to a growth rate, which is appreciably reduced from normal by maintaining a low concentration of one growth factor.

It should, for instance, be easy to determine in what manner oxygen consumption of the bacterial population depends on the growth rate in the chemostat. This can be done by having a closed air circulation through the growth tube, absorbing out the carbon dioxide and determining the pressure change in the system.

A preliminary experiment carried out in the chemostat with the "fast" tryptophane requiring strain seems to show that even when the growth rate of the bacteria is reduced to less than 1/6 of

its normal value ($\frac{V}{W}$ = 6 hrs.) no significant amount of ornithine, citrulline, or arginine are poured out by the bacterial population in the growth tube. Tentatively this suggests that at this lowered growth rate, the synthesis of these other amino acids might perhaps be reduced to a small fraction of what it would be at the normal growth rate.

Further information on bacterial regulations will be furnished by experiments of the following type. The tryptophaneless strain is grown in the chemostat with a concentration of $\underline{a}$ = 200 γ/l in the storage tank and at about $\frac{V}{W}$ = 6 hrs. The bacterial concentration in the growth tube in the stationary state is then about 10^8/cc. If the flow rate is now suddenly changed to correspond to say $\frac{V}{W}$ = 2 hrs., the tryptophane concentration in the growth tube will instantly begin to rise. In the stationary state, corresponding to $\frac{V}{W}$ = 2 hrs., the bacterial concentration is only very slightly lower than in the stationary state corresponding to $\frac{V}{W}$ = 6 hrs. Therefore, if the bacteria were able instantly to adjust their growth rate to the currently prevailing tryptophane concentration, the concentration of the bacteria should not appreciably change upon the changing of the flow rate. If, however, the bacteria, having grown for a period of time at $\frac{V}{W}$ = 6 hrs., cannot instantly adjust their growth rate, we should expect, upon changing the flow rate to $\frac{V}{W}$ = 2 hrs., the bacterial concentration first to fall off, then after a period of time (during which the bacteria adjust), begin to rise again and ultimately to return to about the initial value.