Importance of the Generation Time in Microbiological Experiments

S. HORVÁTH

Institute of Genetics, Hungarian Academy of Sciences, Budapest

Received October 2, 1969

ABSTRACT. The bacterial and tissue cells can grow in batch and continuous culture. In batch culture the cells have different physiological states during incubation. The generation time changes from time to time during growth, except in the exponential phase. In continuous culture the cell growth takes place under steady-state conditions. In different steady-states the generation times reached remain constant at a certain level. This paper presents evidence of how the generation time influences the cell size, the chain formation, the multiplication of viruses, the development of competence both in transformation and transfection and the quantitative changes of lytic factor. In the experiments it is necessary to give the values of the generation times. This parameter helps the experimenters to compare the results and to avoid some errors in their conclusions.

There are a lot of variables during growth. In a particular medium the cell size and composition changes in a characteristic manner during incubation (Hershey & Bronfenbrenner, 1938).

The shorter the generation time, the larger the cell size (Ecker-& Schaechter, 1963), and the higher the number of nuclei per cell. The variation in mass per cell is due to changes in the number of nuclei per cell.

The rate of protein synthesis per unit ribonucleic acid (RNA) is nearly independent of the generation time. The protein fraction must vary greatly in composition if the bacterial cells multiply at different generation times. The protein may change qualitatively. Induction or repression of enzymes may occur during growth. The longer the generation time the greater the ratio of ribosomal RNA to deoxyribonucleic acid (DNA) changes at different generation times (Maaløe & Kjeldgaard, 1966).

In the late exponential growth the proteolytic activity is increased. The specific activity of fumarase and alanine dehydrogenase increase after the end of exponential growth (Warren, 1968). Amylase production in *Aspergillus oryzae* depends upon the growth rate (Meyrath, 1965).

This paper presents evidence of how the microbiological results depend upon the generation times. The following problems are analysed: correction of the viable count, bacterial growth in batch and continuous culture; the influence of the generation time on cell size and on chain formation, on the number of phage-resistant spontaneous mutants, on the multiplication of viruses, on the development of competence both in transformation and transfection, on the quantitative changes of lytic factor. Finally, the significance of the generation time in the microbiological experiments is discussed.

MATERIALS AND METHODS

Bacteria and viruses. Bacillus subtilis Marburg, 168 *try*$^-$, 168M *try*$^-$ *phs* (SPO-1 phage-sensitive), 168M *try*$^-$ *phr* (SPO-1 phage-resistant), SB25 *try*$^-$ *his*$^-$ bacterial strains were used.

SPO-1 subtilis phage and the Aujeszky (pseudorabies) virus strain marked XXV (Horváth, 1959) were used in the experiments.

Tissue culture. Chicken heart fibroblast tissue culture was prepared and maintained in the rolling drum according to Horváth's method (Horváth, 1954; Horváth & Balázs, 1958).

Media. The bacterial strains were maintained in minimal glucose yeast (MGY) liquid medium. T medium was used for transformation and MG agar for the selection of transformants. The composition of the media are described by Horváth (1967).

The composition of the media used in tissue culture is found in an earlier paper (Horváth, 1959).

Cell lysis was assayed in a buffer-salt solution (Horváth, 1968b).

DNA preparation. Transforming DNA of *Bacillus subtilis* Marburg was prepared by the phenol extraction method of Saito and Miura (1963). The infectious phage DNA was prepared from the concentrated SPO-1 phage suspension by the phenol extraction method of Mandell and Hersey (1960).

Cultivation of the bacterial suspension. The bacterial experiments were carried out in 100 ml Erlenmeyer flasks fitted with a side arm for optical density (OD) measurements. The bacterial cells were suspended in 10 ml MGY liquid medium and were shaken in a water bath at 37 C. During incubation the bacterial multiplication was followed by a densitometer. The generation time was found to be exactly the mass doubling time (τ_d).

RESULTS

Correction of the viable count

The bacterial cell count is important, because it permits the calculation of the generation time and the quantitative study of bacterial metabolism.

A useful method for counting the total number of organisms is the opacity method. It is widely used at the present time. The OD is evaluated not only by bacterial numbers but also by their size. The OD gives good information on the total amount of bacterial protoplasm.

According to the plating method the average number of colonies per plate in a given dilution is the basis for estimating the number of colony-forming units (c.f.u.).

Most of the bacterial strains used in the experiments form cell-aggregates and the cells remain attached to one another by their cell-membranes after their divisions (chain -formation, irregular groups of cocci etc.).

When the exact number of living bacterial cells needs to be determined in a chain-forming bacterial strain, a correction must be made, which is calculated from the c.f.u. in the following way. Smears of the bacteria are examined by the light microscope. The different length of chains (including single cells) are counted in some fields and the results are analysed statistically. The ratio of the total number of cells to the number of chains (including single cells) gives a factor. By multiplying the c.f.u. by this factor, the corrected value is obtained, which shows the exact number of single cells found in the population.

Bacterial growth in batch and continuous culture

(A) In batch culture the generation time changes from time to time during growth. Only in the exponential phase does it remain nearly constant. Depending on the cultural conditions the doubling time may be the following: (1) the division time of individual cells is the individual generation time or the interdivision time; (2) the arithmetic average of the individual generation time is the mean generation time or the average interdivision time; (3) the number doubling time or the mean effective generation time (τ_e); (4) the mass doubling time (τ_d).

The following experiment shows how the mass doubling time changes in the same bacterial populations during incubation using different kinds of glassware and cultural conditions. An overnight culture of *Bacillus subtilis* 168M

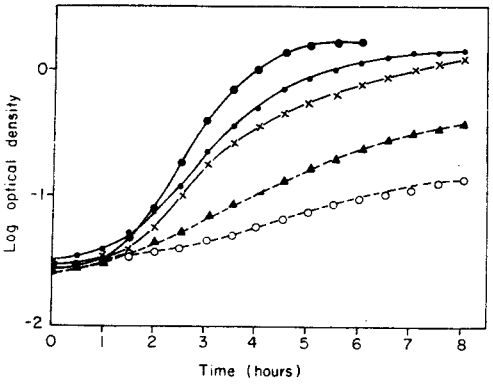

Fig. 1. The growth curves of 5 ml *B. subtilis* 168M *try⁻ phs* cell suspensions cultivated in different conditions at 37 C. ●—● — in a 100 ml Erlenmeyer flask, shaking; · — · — in a 100 ml Erlenmeyer flask, in horizontal position; + — + — in a 50 ml Erlenmeyer flask, in horizontal position; ▲---▲ — in a tube, in oblique position; ○---○ — in a tube, in vertical position.

try⁻ was suspended in MGY liquid medium to 0.025 OD. 5 ml of this bacterial suspension were measured into different kinds of glassware. During incubation at 37 C the ODs were followed and the results are shown in Fig. 1.

From the growth curves the mass doubling times were determined in each case (Fig. 2).

In the Figures it is seen that there are big differences among the growth curves and among the doubling times as well.

The growth of an exponentially growing bacterial suspension may be expressed by the following equation:

$$\frac{dn}{dt} = v_m n \qquad (1)$$

or

$$n = n_0 e^{v_m(t-t_0)}, \qquad (2)$$

where n is the number of bacteria at time t, n_0 is the number of bacteria at

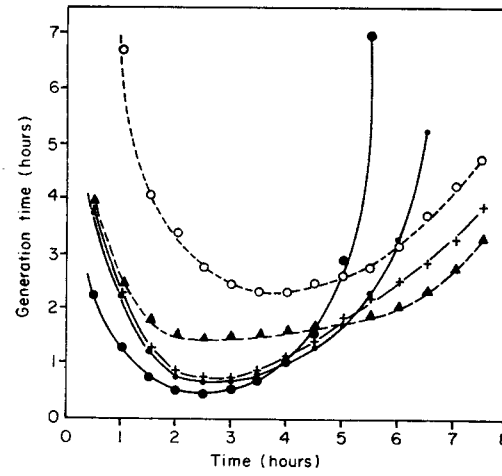

Fig. 2. The generation time curves of 5 ml *B. subtilis* 168M *try⁻ phs* cell suspension cultivated in different conditions at 37 C. Details of explanations in Fig. 1.

time 0, v_m is the growth rate constant and t is time. Taking logarithms of equation 2 yields

$$\ln n = \ln n_0 + v_m(t - t_0). \qquad (3)$$

The growth may be characterized by τ_e, the number doubling time.

$$\ln 2n_0 = \ln n_0 + v_m\tau_e \qquad (4)$$

and

$$\tau_e = \frac{\ln 2}{v_m} \qquad (5)$$

It is convenient to distinguish the mass doubling time, τ_d, which is equal to the following equation:

$$\tau_d = \frac{\ln 2}{\mu_m}, \qquad (6)$$

where μ_m is the mass growth rate constant (Powell, 1956).

(B) In continuous culture the experimenter can control the flow rate or the bacterial density depending upon what kind of apparatus is used; the chemostat (Novick & Szilard, 1950; Monod, 1950) or the turbidostat (Bryson & Szybalski, 1952).

In continuous culture the net rate of

increase of concentration of bacterial cells is given by the following equation:

$$\frac{dx}{dt} = \mu x - Dx\,, \qquad (7)$$

where x is the concentration of bacteria in the vessel, μ is the specific growth rate and D is the dilution rate (Herbert,

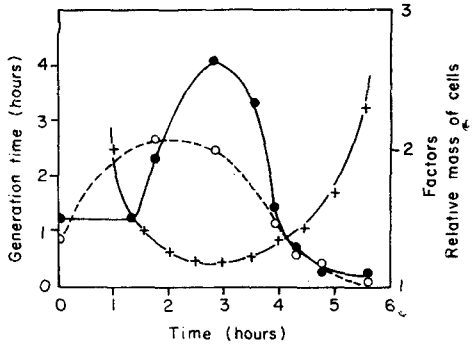

Fig. 3. The values of the relative cell size and the correction factor in *B. subtilis* 168 *try*⁻ cell suspension multiplied in different generation times. ●—● — relative cell size; ○---○ — correction actor; + — + — generation time.

Eisworth & Telling, 1956). When $\mu = D$, $dx/dt = 0$, and x is constant; steady-state of growth occurs. It means that the concentration of bacterial cells and substrates does not change during incubation, but remains constant and the population must be growing exponentially. Between zero and the critical value of the dilution rates a large number of steady-states can be obtained (Monod, 1942; 1950).

Influences of the generation time on cell size and on chain formation

Bacillus subtilis 168 *try*⁻ was cultivated in MGY medium and the correction factor was determined, because this strain formed chains. The relative size of the cells was also calculated (Fig. 3).

In Fig. 3 it is seen that there are some correlations between generation time and both the correction factor and the relative cell size. The shorter the generation time the higher the values of both the correction factor and the relative cell size.

Influence of the generation time in the number of phage-resistant spontaneous mutants

To determine the number of phage-resistant spontaneous mutant *Bacillus subtilis* 168M *trv*⁻ *phs* strain was cultivated in MGY medium. During growth, samples were taken and adjusted to 0.025 OD with MGY medium. To 1 ml of this suspension 1 ml MGY medium containing 5.4×10^8 SPO-1 phage particles was measured. After 10 min incubation at room temperature it was plated on MGY agar. The phage-resistant colonies were counted the following day (Fig. 4).

It is seen that the shorter the generation time the smaller the number

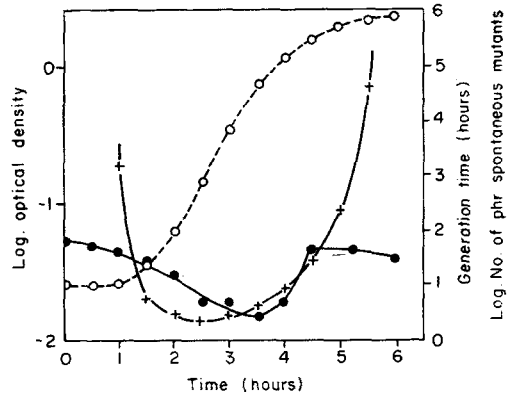

Fig. 4. Changes in the number of SPO-1 phage-resistant spontaneous mutant in *B. subtilis* 168M *try*⁻ *phs* cells during incubation. ●—● — spontaneous mutants; ○---○ — OD; + - - - + — generation time.

of spontaneous phage-resistant mutants. This correlation is easy to understand because the bacterial cells are more sensitive to the phage particles when they grow well.

Bacteriophage and virus multiplication in cells multiplying at different generation times

In the following experiments the SPO-1 phage and the virus of Aujeszky's disease multiplication were investigated:

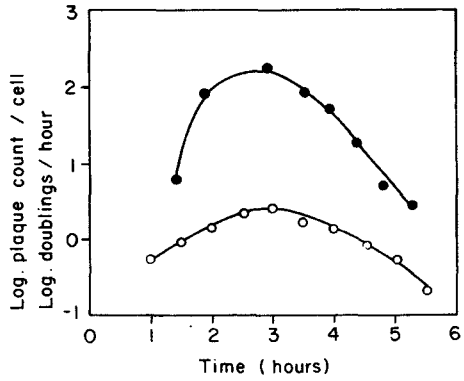

Fig. 5. The relation between doubling per hour and the BS in *B. subtilis* 168M *try⁻ phs* infected by SPO-1 phage. ●—● — BS; ○—○ — doublings per hour.

(a) *Bacillus subtilis* 168M *try⁻ phs* cells multipling at different generation times were infected by SPO-1 phage at an average multiplicity of 1 phage per bacterium. One step growth experiment was carried out and the burst size (BS) was calculated. The results and the values of doublings per hour (reciprocal value of generation time) are shown in Fig. 5.

The characteristics of the curves are nearly the same and the peaks were found at the same time. Thus the shorter the generation time the higher the BS.
(b) Aujeszky virus was used in the next experiment. The fibroblast tissue cultures were precultivated for varying periods of time, having different generation times before the virus inoculation. The titers of the same virus suspension were different depending upon the time of precultivation. The results of the experiment carried out in 15 parallel tubes per dilution are seen in Fig. 6.

The highest titer was obtained when the generation time was the shortest.

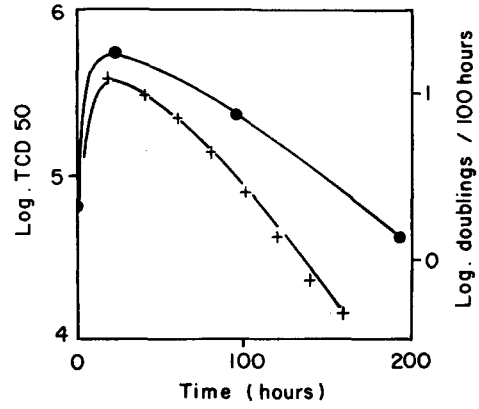

Fig. 6. Titers of Aujeszky virus suspension using chicken heart fibroblast culture having different generation times at the time of inoculation. ●—● — TCD 50 titers of virus; + — + — doublings per 100 hours.

Influence of the generation time in the development of competence in transformation and transfection

(a) Many bacterial species are able to take up DNA when in a state of "com petence" and can be transformed (Ravin, 1961; Braun, 1965). In the transformation experiment the recipient strain was *Bacillus subtilis* 168M *try⁻ phr*. The transforming DNA was isolated from *Bacillus subtilis* Marburg. The bacterial cells were maintained in MGY medium. During growth, samples were taken and

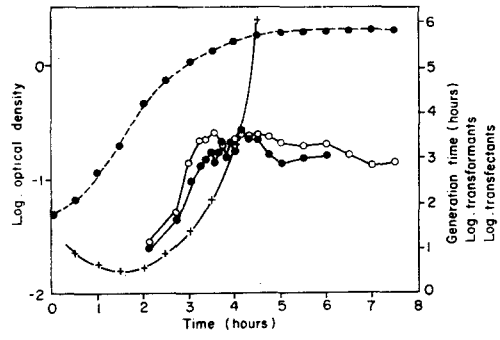

Fig. 7. Number of transformants and transfectants in *B. subtilis* 168M *try⁻ phr* suspension during incubation. ●···● — OD; ○—○ — transformants; ●—● — transfectants; +—+ — generation time.

assayed for the number of *try*$^+$ transformants (Fig. 7).

In Fig. 7 it is seen that the generation time played an important role in the development of competence, which followed a definitive course during incubation. It is clear that there is some regularity in the development of competence, and it is related to the changes in generation time. The peak of competence develops in the late exponential phase of growth when the bacterial cells multiply at a certain rate, when the mass doubling time is about 1 hour 40 minutes.

(b) In transfection the competent phage-sensitive and phage-resistant cell surface as well are able to adsorb the infectious phage DNA molecules and in both cases infective phage particles are produced (Romig, 1962; Okubo, Strauss & Stodolsky, 1964; Reilly & Spizizen, 1965).

In transfection, *Bacillus subtilis* 168M *try*$^-$ *phr* cell suspension multipling at different generation times was adjusted to 0.1 OD with MGY medium. 0.25 ml of this cell suspension was measured to 0.25 ml SPO-1 phage DNA to give a final concentration of about 10 μg DNA/ml. The mixture was then shaken for 90 min in a water bath at 37 C and the plaque forming units were determined on a plastic tray (Horváth & Alföldi, 1954). The results are demonstrated in Fig. 7.

It seems that the curve of competence is nearly the same as that in transformation. The peak of competence was obtained in the late exponential phase of growth, where the highest number of transfectants were found, and the bacterial cells multiplied at a generation time of about 2 hours.

Relationship between the activity of the lytic factor and the generation time

Young and Spizizen (1961; 1963) demonstrated the presence of a lytic factor in the wall of competent *Bacillus subtilis* cells. In the following experiment the activity of the lytic factor is demonstrated in the cells multipling at different generation times. *Bacillus subtilis* SB25 *try*$^-$ *his*$_2^-$ was shaken in MGY liquid medium, at 37 C. During growth, samples were taken and centrifuged, then re-

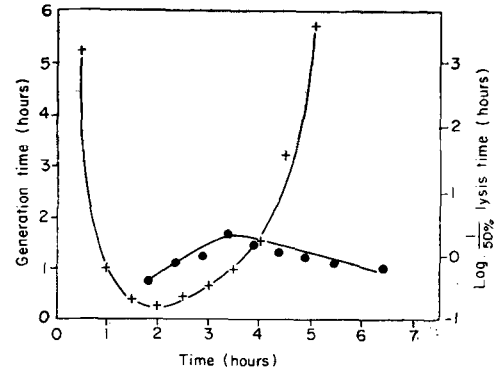

Fig 8 The activity of the lytic factor in *B subtilis* SB25 *try*$^-$ *his*$_2^-$ during incubation. ●—● — activity of the lytic factor; + — + — generation time.

suspended in balanced salt solution (Horváth, 1968b) to 0.4 OD and was shaken in a water bath at 37 C. The lysis was estimated by controlling the change in OD. The reciprocal value of the 50% lysis time in hours was regarded as the activity of the lytic factor (Fig. 8).

The peak of the activity of the lytic factor occurred when the generation time was of about 1 hour 30 min.

Importance of the generation time in the microbiological experiments

If the generation time is known the other parameters can be calculated so that growth can be described precisely. For the determination of the generation time it is important to count the number of bacterial cells with a certain exactness during incubation. Often, it is difficult to do this because the bacterial strains form cell-aggregates. Bacterial chains, groups of cocci etc. mainly influence the precise determination of the number of cells. The opacity method expresses only

the mass of the cells. For this reason it is necessary to make a correction whenever it is possible.

All the physiological states of the cells are important either from scientific or practical points of view. Using batch culture it is possible to determine the curve of a certain biological condition concerning different generation times. In batch culture the exponential growth takes only a short period of time, depending upon the size of the inoculum. In a semilogarithmic plot the exponential growth is nearly a straight line, but the level of energy and the physiological state of the cells are different along this line. At the beginning of the exponential growth the cells divide as fast as they can, depending upon the cultural conditions.

In expressing the level of a physiological state it is necessary also to give the values of the generation times in all cases.

Calculating the rate of mutation of phage-resistant spontaneous mutants (Luria & Delbrück, 1943), it is necessary to give the generation time when the cells are infected by the phage particles.

In virus titration the shorter the generation time the higher the infective titer and the BS. A big difference may be found in the infectious titers of a virus suspension when the cells are infected at different generation times.

The competence in transformation and transfection exist when the bacterial cells multiply at a certain rate. The cells multipling in the shortest generation time cannot be transformed or transfected. The competence can be "fixed" at an optimal level in balanced growth. A different level of competence can be maintained in a steady-state of growth concerning the values of the generation time (Horváth, 1968a).

The activity of the lytic factor was determined in different *Bacillus subtilis* strains. The highest activity was found at about 1 hour 40 min generation time in *Bacillus subtilis* 168 and 168M *try⁻ phs* strains (Horváth, 1968b). At the shortest generation time the activity of the lytic factor was very low.

The steady-state of growth is important when it is desired to maintain the cell culture at an optimal level of a certain physiological state. It is useful not only in scientific research, but also in practice. A lot of steady-states of growth can be maintained at different generation times and in this state the number or the mass of the cells are constant during growth. Different generation times are obtained in steady-state of growth by regulating the quantity of the inflowing medium (D value) or the density of the cell suspension.

In general we cannot say that a short or the long generation time is the best. Optimal level of a physiological state exists at a certain value of the generation time.

In any case at all physiological states of bacterial cells that exist in reality may be important from the experimental point of view.

In order to compare the results obtained in different laboratories it is necessary to give the value of the generation time so that the experimenters may avoid some errors in their conclusions.

The author wishes to thank Dr. B. Györffy for valuable criticism and Miss I. Kállay for skilled technical assistance.

References

Braun, W.: *Bacterial genetics*, p. 239. W.B. Saunders Company, Philadelphia & London, 1965.

Bryson, V., Szybalski, W.: *Microbial selection.* Science, 116 : 45, 1952.

Ecker, R. E., Schaechter, M.: *Bacterial growth under conditions of limited nutrition.* Ann. N. Y. Acad. Sci. 102 : 549, 1963.

Herbert, D., Eisworth, R., Telling, R. C.: *The continuous culture of bacteria; a theoretical and experimental study.* J. gen. Microbiol. 14:601, 1956.

Hershey, A. D., Bronfenbrenner, J.: *Factors limiting bacterial growth.* J. gen. Physiol. 21 : 721, 1938.

Horváth, S.: *A new sensitive method of the rolling drum type for influenza virus titration.* Acta Microbiol. Acad. Sci. hung. 1 : 481, 1954.

Horváth, S.: *A quantitative semi-micro tissue culture method and its use in microbiology II. Use of the method in titrating the virus of Aujeszky disease.* Acta Microbiol. Acad. Sci. hung. 6 : 5, 1959.

Horváth, S.: *Development of competence in cultures of Bacillus subtilis inoculated with different numbers of bacteria.* J. gen. Microbiol. 48 : 215, 1967.

Horváth, S.: *Competence in Bacillus subtilis transformation system.* J. gen. Microbiol. 51 : 85, 1968a.

Horváth, S.: *Lytic factor and competence in Bacillus subtilis.* Acta Microbiol. Acad. Sci. hung. 15 : 173, 1968b.

Horváth, S., Alföldi, L.: *A new and sensitive method of phage titration on plastic trays.* Acta Microbiol. Acad. Sci. hung. 1 : 495, 1954.

Horváth, S., Balázs, V.: *A quantitative semi-micro-tissue culture method and its use in microbiology I. Quantitative semi-micro tissue culture method.* Acta Microbiol. Acad. Sci. hung. 5 : 415, 1958.

Luria, S. E., Delbrück, M. E.: *Mutation of bacteria from virus sensitivity to virus resistance.* Genetics, 28 : 491, 1943.

Maaløe, O., Kjeldgaard, N. O.: *Control of macromolecular synthesis.* W. A. Benjamin, Inc. New York, Amsterdam, 1966.

Mandell, J. D., Hershey, A. D.: *A fractionating column for analysis of nucleic acids.* Anal. Biochem. 1 : 66, 1960.

Meyrath, J.: *Interdependence of inoculum size, method of cultivation and substrate composition in amylase production and growth of Aspergillus oryzae.* Zbl. Bakt. Parasit. Inf. Hyg. 119 : 53, 1965.

Monod, J.: *Recherches sur la croissance des cultures bacteriennes.* Paris. Herman & Cie. 1942.

Monod, J.: *La technique de culture continue, théorie et applications.* Ann. Inst. Pasteur 79 : 390, 1950.

Novick, A., Szilard, L.: *Description of the chemostat.* Science 112 : 715, 1950.

Okubo, S., Strauss, B., Stodolsky, M.: *The possible role of recombination in the infection of competent Bacillus subtilis by bacteriophage deoxyribonucleic acid.* Virology 24 : 552, 1964.

Powell, E. O.: *Growth rate and generation time of bacteria, with special reference to continuous culture.* J. gen. Microbiol. 15 : 492, 1956.

Ravin, A. W.: *The genetics of transformation.* Adv. Genetics 10 : 61, 1961.

Reilly, B. E., Spizizen, J.: *Bacteriophage deoxyribonucleate infection of competent Bacillus subtilis.* J. Bacteriol. 89 : 782, 1965.

Romig, W. R.: *Infection of Bacillus subtilis with phenol extracted bacteriophages.* Virology 16 : 452, 1962.

Saito, H., Miura, K. I.: *Preparation of transforming deoxyribonucleic acid by phenol treatment.* Biochim. biophys. Acta 72 : 619, 1963.

Warren, S. C.: *Sporulation in Bacillus subtilis. Biochemical changes.* Biochem. J. 109 : 811, 1968.

Young, F. E., Spizizen, J.: *Physiological and genetic factors affecting transformation of Bacillus subtilis.* J. Bacteriol. 81 : 823, 1961.

Young, F. E., Spizizen, J.: *Biochemical aspect of competence in the Bacillus subtilis transformation system.* J. biol. Chem. 238 : 3126, 1963.