FACTORS LIMITING BACTERIAL GROWTH

III. Cell Size and "Physiologic Youth" in Bacterium coli Cultures

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It has been repeatedly shown that the metabolic activity in a bacterial culture computed on a rate per cell basis increases during the period of lag to a peak somewhat before the maximum rate of multiplication is attained, and afterward falls off to a very small value. This finding usually has been regarded as evidence that bacteria undergo rejuvenescence prior to active multiplication. Although the underlying observations have been amply confirmed, this interpretation has seemed to us open to question.

The concept of physiologic youth, originally applied to bacteria by Sherman and Albus (1) to signalize their observation that during the period of rapid multiplication bacteria exhibit increased susceptibility to injurious influences, has since become established as the most satisfactory interpretation of the fact that bacteria transferred to sterile broth multiply for a time at a rate which is dependent on the condition of the culture from which they are taken. The observations of Clark and Ruehl (2), and of Henrici (3), concerning the systematic changes in morphology and size of individual cells during the cycle of growth, gave particular credence to this view. Apart from the fact that it is based on doubtful analogy with mammalian physiology, the hypothesis of cellular differentiation has remained of limited usefulness especially because the experiments designed to demonstrate differences in the metabolism of "embryonal," mature, and senescent forms of bacteria, have not been entirely convincing. This has been due to failure to control adequately some variables known to influence the physiology of bacteria in artificial culture.

In some cases the size of individual cells or groups of cells has been left out of account (4), and nearly always the influence of the changing environment itself has been disregarded (4–6). Nevertheless, the observed differences in physiological activity of the cells often have been attributed to differences within the cell itself.

Only a few investigators have realized the necessity of eliminating the effects due to changing external environment. Thus in the original observations of Sherman and Albus (1) on resistance, for example, environmental factors were controlled to the extent that uniform populations were employed. More recently Longsworth and MacInnes (7) were able to maintain a constant pH and the gaseous environment in actively growing cultures of *Lactobacillus acidophilus* and found that under these conditions during the latter part of the growth cycle, while generation time showed a tendency to increase, the fermentation rate per cell was markedly decreasing. Also Moyer (8), who studied cataphoretic mobility of bacteria suspended in buffer solutions, found differences which were related in a consistent manner to the phase of growth of the parent culture. He believed that these differences could not be attributed to variations in the size of the cells. More recently Wooldridge, Knox, and Glass (9) have studied the metabolic activities of non-proliferating bacteria under uniform conditions in relation to the age of the parent culture. These authors found that, among cell suspensions of equal turbidity prepared from cultures of different age, those derived from 24 hour cultures showed maximal activity irrespective of the nature of the substrate. Although the phase of growth of these cultures was not indicated, it is difficult to relate this finding to the frequent observation that heightened activity occurs at the end of the period of lag. When the relation between metabolic activity and viable count was studied by the same authors (10), greater discrepancies were found, but again correlation with phase of growth was not made. In the authors' opinion, these differences could not be attributed to variations in the size of cells.

Clifton (11) has expressed the contrary opinion, for which his experiments have provided only indirect evidence, namely that the metabolic activities of the cells in a bacterial culture are influenced primarily by their size, and by the changing conditions of their

growth. Our own preliminary experiments seem to have justified a similar conclusion (12).

The experiments to be described in this paper were designed to measure the physiologic changes which bacteria undergo during the various phases of multiplication, with particular attention to controls suggested by the considerations outlined above. The manometric method which was chosen for this purpose was found to yield reliable information as to the rate of oxygen uptake of cells of *Bacterium coli* removed from cultures after various intervals of growth and placed in a uniform medium favorable to continued growth, and to permit the correlation of these data with both the number of viable cells and the total mass of bacterial substance.

The bacteria were cultivated in filtered beef extract broth at 37°C. in Erlenmeyer flasks filled to a depth of 2 cm., removed from this medium by rapid centrifugation, and suspended without washing in 0.85 per cent saline. It was found that when 100 ml. of the medium were seeded with 0.01 ml. of an 18–24 hour culture grown in the same medium, reproducible growth curves were obtained, thus indicating that the phase of growth of cultures of a given age could be predicted. The age of the culture was reckoned as the interval between seeding and suspending the centrifuged cells in saline (8). Uniform bacterial content was obtained by suitable dilution of the saline suspension on the basis of nephelometric estimation of bacterial substance. Viable counts were made by the dilution and pour-plate method either from the saline suspension or, in many experiments, from one of the vessels of the respirometer. The population of the original culture was obtained from the viable count of the saline suspension by computing to original volume. Oxygen consumption was measured in the Barcroft-Warburg apparatus at 37°C., with 3 aliquots estimated from the turbidity of the original suspension to contain respectively the nephelometric equivalent of 10^8, 3×10^8, and 10^9 organisms from a 24 hour culture. The cell suspension was placed in the vessel with sufficient 0.85 per cent saline to bring the total volume to 4 ml., the sidearm containing 1 ml. of beef extract medium of such composition that, after mixing, concentrations of peptone 1.0 per cent, beef extract 0.5 per cent, and phosphate buffer of pH 6.6 M/50 were obtained. The nutrient mixture was added to the bacterial suspension after temperature equilibrium was established (usually requiring 10 minutes), the zero time noted, and the viable count made. The readings of the manometers were continued at 5 minute intervals for approximately 1 hour. Values for total bacterial substance in the saline suspension were obtained both by means of a photoelectric nephelometer (13), and by determination of bacterial nitrogen by the micro-Kjeldahl method, after a second centri-

fugation to remove soluble nitrogen. In blank determinations, made by adding small numbers of bacteria of known nitrogen content to fresh broth and recentrifuging, no increase in nitrogen was observed.

The average rates of oxygen uptake during the successive 5 minute intervals were spotted at the mid points of corresponding intervals on the graph, and the points connected by a curve smoothed, if necessary, by inspection. Usually the rate for the second, and often for the first 5 minute interval fell on this line. The error introduced by assigning the average rates to the mid points of the 5 minute intervals was too small to be significant. By extrapolation of this curve to the ordinate, the rate of oxygen consumption at zero time, corresponding to the moment at which the substrate was added, was obtained. This value has been used in all calculations.

The rates of oxygen uptake at zero time per milliliter of the original suspension for the three measurements showed satisfactory agreement. Their average divided by the numbers obtained from the viable count, and by the nitrogen content, respectively, gave the rate of oxygen uptake "per viable cell," and "per gram of bacterial nitrogen."

Turbidity was measured by dilution of the suspension to give the zero reading at the resistance box when matched against a 0.5 per cent solution of copper sulfate as standard. The ratio between the average number of viable cells in suspensions from 18–24 hour cultures, after dilution to standard turbidity, and the numbers in the test suspension similarly diluted, expressed the "nephelometric index of size." By dividing the rate of oxygen uptake per cell by this index, the rate "per nephelometric cell" was obtained.

For example, a 3 hour culture is centrifuged and the cells are suspended in 1/20 volume of saline. The following data are obtained:

Dilution in saline required to match 0.5 per cent $CuSO_4$ in the nephelometer =	1:8.6
Numbers of 18–24 hr. cells giving similar turbidity =	3.7×10^7/ml.
$-O_2$ per hour for: 3.1 ml.	181.2 mm.^{3}
0.94 "	54.9 "
0.31 "	17.9 "
1.0 " (average)	58.2 "
Nitrogen content	1.24×10^{-2} mg./ml.
Viable count	6.0×10^7/ml.

Calculations are made as follows:

Population of parent culture— $\frac{6.0 \times 10^7}{20} = 3.0 \times 10^6$ bacteria/ml.

$-O^2$ per cell hour— $\frac{58.2}{6 \times 10^7} = 9.7 \times 10^{-7}$ mm.^{3}

$-O^2$ per gm. N per hr.— $\frac{58.2}{1.24 \times 10^{-5}} = 4.7 \times 10^6$ mm.^{3}

Nephelometric index of size— $\frac{3.7 \times 10^7}{6/8.6 \times 10^7} = 5.3$

$-O_2$ per nephelometric cell per hour— $\frac{9.7 \times 10^{-7}}{5.3} = 1.8 \times 10^{-7}$ mm.^{3}

Nitrogen per cell— $\frac{1.24 \times 10^{-2}}{6.0 \times 10^7} = 2.1 \times 10^{-10}$ mg.

The results of the individual measurements are shown in the figure where they are plotted against the age of the parent cultures. Averages of these values for the several time intervals are given in the table. The values indicated on the graph for cultures at zero time represent the mean of the corresponding values for 18 to 24 hour cultures (*i.e.*, of the cells used for seeding), which accordingly appear twice in the graph. No measurements could be made until populations approaching 10^7 cells per milliliter were reached, which occurred near the end of the phase of lag. This probably corresponds with the point of maximum size and activity per cell, as observed by Martin and others (4–6) in proliferating cultures. In several trials with cultures seeded with larger numbers, and examined at $1\frac{1}{2}$ to 3 hours, the activity per viable cell was always much less than the highest values recorded in the table. This finding is in conformity with the observation of Henrici (3), that the maximum size attained decreases as the size of seeding is increased.

The curve correlating oxygen uptake with bacterial numbers shows clearly that cells taken from cultures just entering the phase of logarithmic multiplication consume oxygen at six times the minimum rate prevailing near the end of this period. There is no further change as the culture ages. Thus our observations with cell suspensions confirm the findings of previous investigators (4–6), who have used growing cultures, only with respect to the early phases of growth.

The Average Size of Cells and Rate of Oxygen Uptake in Relation to Age of Cultures of Bacterium coli

1	2	3	4	5	6	7	8	9	10	11
No. of observations	Age of parent culture	Population of parent culture × 10^6/ml.	Gen.*	GO_2†	$-O_2$ per cell hr. × 10^{-7} mm.³	$-O_2$ per gm. N per hr. × 10^5 mm.³	$-O_2$ per nephelometric cell per hr. × 10^{-7} mm.³	N per cell × 10^{-10} mg.	Nos.‡ giving equal turbidity × 10^7/ml.	Nephelometric index of size
	hrs.		*per hr.*	*min.*						
3	3	2.8	3.0	35	11.1	5.0	2.1	2.2	0.7	5.3
5	4	36	3.5	35	7.0	4.9	2.0	1.5	1.0	3.7
6	5	220	2.2	35	3.8	4.9	2.0	0.80	2.1	1.9
3	6	450	1.0	35	1.7	4.5	1.7	0.38	3.7	1.0
3	8	830	0.2	35	2.0	4.6	2.0	0.43	3.7	1.0
3	12	1100	<0.1	35	2.1	4.8	1.8	0.42	3.2	1.3
4	18	1300	<0.1	35	2.1	5.1	2.3	0.41	4.1	0.9
4	24	1400	<0.1	35	1.8	5.1	1.6	0.35	3.4	1.1

* Rate of multiplication in the parent culture by graphic interpolation from values given by the formula $n/t = \frac{\log b - \log B}{t \log 2}$: where B = initial, and b = final numbers, for the observed interval t.

† Time required for twofold increase in rate of oxygen uptake in sub-culture.

‡ Expressed as numbers per milliliter of saline matching 0.5 per cent $CuSO_4$.

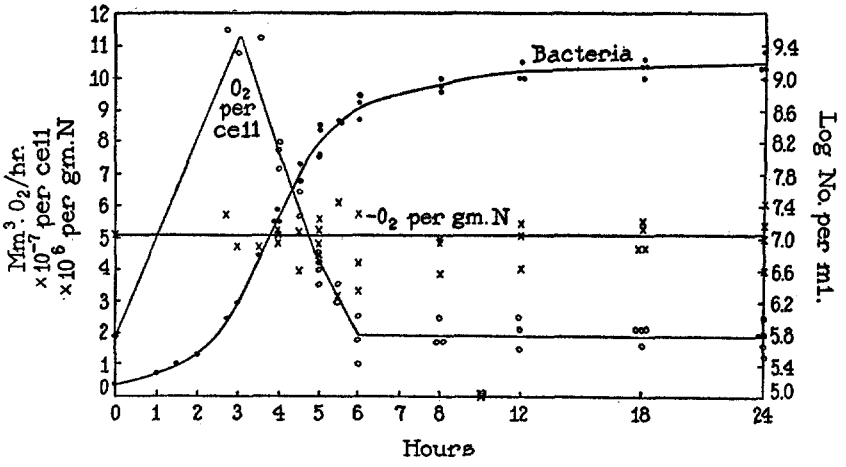

FIG. 1. Rates of oxygen uptake per viable cell, and per gram of bacterial nitrogen, in relation to phase of growth of *Bacterium coli* cultures.

The continuous decrease in metabolic rate per cell observed by them during the later periods does not appear in our data. We believe that in these earlier experiments the fall in the metabolic rate below the initial value must be attributed to changes in the medium during growth, and to competition within the growing population, especially for oxygen (11–12), and in no way reflects changes in the cells themselves. In our measurements, which were carried out with similar populations in mediums of uniform composition, secondary effects due to changes in environmental conditions have been excluded.

Very different are the results obtained when rates of oxygen consumption are computed in terms of the unit of bacterial nitrogen. On this basis, values for oxygen uptake are constant throughout the period of observation. This suggests that the significant variable in cultures of different age is the average size of cells. Column 9 of the table shows the magnitude of these changes as expressed by the "nitrogen per cell" quotient. This quantitative agreement between changes in oxygen consumption per cell, and the average cell size, may be taken as sufficient evidence that the one is wholly referable to the other. The size indices (column 11) obtained from the nephelometric data are in good agreement with the nitrogen per cell values and lead to the same conclusion.

It is interesting that the time required for a twofold increase in rate of total oxygen uptake of the culture (corresponding to a functional "generation time"), is the same for cells of all ages, as can be seen in the table. This observation suggested another type of experiment (14) in which it was found that multiplication rates of *Bacterium coli* during the early hours of growth are in a large part the expression of changes taking place in the average size of cells. In particular, the phenomenon of lag seemed to be wholly referable to these changes. The manometric findings suggest further that the absolute growth rate is the same for both young and old cells. This conclusion must be made with caution, however, since evidence has been obtained that deprivation of carbon dioxide in the respirometer cultures imposes a new limiting factor on the rate of growth, at least when small inocula are used.

These experiments have not revealed any specific differences between the metabolism of young and old cells which might be described as physiologic youth and maturity. They do not, of course, explain

other differences, such as cataphoretic mobility (8), and susceptibility to unfavorable environment (1), neither of which, however, in our opinion provides adequate basis for the hypothesis of cellular differentiation.

Subsequent experiments will be directed toward elucidation of the environmental factors influencing the size of cells, and further correlation between the age of the parent culture and the absolute rate of growth of transplants.

SUMMARY

1. Measurements of the rate of oxygen uptake per cell in transplants of *Bacterium coli* from cultures of this organism in different phases of growth have given results in essential agreement with the observations of others.

2. Correlations of viable count, centrifugable nitrogen, and turbidity, with oxygen consumption, indicate that the increased metabolism during the early portion of the growth period is quantitatively referable to increased average size of cells.

3. Indirect evidence has suggested that the initial rate of growth of transplants is not related to the phase of growth of the parent culture.

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