# STUDIES OF CERTAIN FACTORS INFLUENCING THE SIZE OF BACTERIAL POPULATIONS

J. P. CLEARY, PAUL J. BEARD AND C. E. CLIFTON

*Department of Bacteriology and Experimental Pathology, Stanford University, California*

Received for publication, April 13, 1934

Notwithstanding the wealth of investigation carried out on bacterial growth, relatively few consistent data are available on the intermediate phases; i.e., the phase of negative acceleration and closely related to it, the maximum stationary phase. It would be of interest to know why cells suddenly deviate from an orderly logarithmic growth process to one of progressively increasing generation time and why cells cease to multiply before spatial restrictions limit them.

The literature lacks a convincing trend on any experimental basis. That there are conflicts of opinion and data is not surprising when one considers the vagaries of bacterial strains, the diversity of species requirements, and the difficulty of reproducing in one laboratory the exact experimental conditions in another. The present studies and others to follow are undertaken in an effort to reconcile if possible, by repeating and extending in one laboratory with a single strain of organism, investigations in this field. No special effort is made to review the literature. Henrici (1928) and Rahn (1932) have recently filled that need admirably.

Since the physical factors of environment are subject to fairly accurate control, one may profitably turn to those of biochemical nature.

A strain of *E. coli* has been selected for these studies by reason of the more extensive information available on its metabolism and the ease with which contaminants may be detected. After investigation of various counting techniques the plating method was used. Counting error was consistently within 5 per cent.

Investigation to date has been confined to fluid media. This, unless otherwise stated, was a 1.0 per cent peptone, at a temperature of 37.5°C. Sampling was always preceded by agitation or stirring.

Depletion of food supply, accumulation of metabolites, specific autotoxins, growth-inhibiting hormones and physical crowding have all had their supporters. It is entirely possible, too, that combinations of these may operate.

Relatively little consideration and experimentation seems necessary to render some of these conjectures untenable. In point, is the possibility of physical crowding. Henrici, taking exception to this, has pointed out that organisms growing on solid media are tremendously more crowded than in fluid cultures. Two experiments in these laboratories militate against the crowding theory. A peptone suspension containing $1.5 \times 10^9$ organisms killed by exposure to 56°C. in a water bath for one hour, was seeded with a young growing culture of *E. coli*. The control contained a similar number of living coli only. Counts made after fifty hours showed a viable cell count of $100 \times 10^7$ in the overloaded culture and $87 \times 10^7$ in the control. The slight increase is attributed to nutritives made available by the heat treatment of the killed cells. Certainly, there was no evidence of spatial restriction.

The accumulation of metabolites, the formation of specific autotoxins or the development of growth-inhibiting hormones have received support. Undoubtedly toxic wastes do accumulate. Satisfactory evidence of the latter two has yet to be offered.

In one experiment a culture which had passed its growth peak to a practically stationary phase was divided into two equal portions. To one, was added 0.1 ml. of an extract prepared from peptone by Dr. Sahyun in these laboratories. The other portion served as the control. The initial bacterial population was $44 \times 10^6$. At the end of twenty-four hours the control count was $50 \times 10^6$ while that in the flask receiving the extract had increased to $100 \times 10^7$. The nature or function of this extract has not been fully studied yet. That it is not merely a more readily available form of food serving a nutrition function is definitely established.

It may serve in some way to fulfill an energy need. This weakens the case for specific toxins or growth-inhibiting hormones for these if present, should continue to exert their effect in spite of the activator.

In another experiment *E. coli* was grown in a synthetic medium containing ammonium sulphate and pure amino acids as nitrogen sources. After the culture had passed its peak at $400 \times 10^6$ it was divided into two portions. To one was added 1 cc. of 2 per cent peptone and to the other an equivalent amount of nitrogen as synthetic medium. Counts after twenty-four hours showed a population of $800 \times 10^6$ per cubic centimeter, in that receiving peptone and $490 \times 10^6$ in that receiving the synthetic medium.

It cannot be denied that the accumulation of metabolites acts to restrict bacterial activity. But the rôle of the acknowledged toxic wastes in many cases has not been satisfactorily disengaged from other "possible factors." That is, certain phenomena may operate to restrict growth before wastes become significant. The effect of toxic substances present might then be enhanced. Depletion of food, exhaustion of energy sources or both might conceivably be such factors.

Some evidence in this direction is offered by an experiment in which 1.0 ml. of 10 per cent peptone was added daily to a culture whose initial volume was 100 ml. Additions were begun fifty-four hours after the inoculation when the culture had passed its growth peak of $124 \times 10^7$ and declined to $107 \times 10^7$. The results with the control are included in table 1. In a similar experiment, half the volume was withdrawn each day and replaced with 1 per cent peptone. These data confirm those of Graham Smith (1920). It would appear that depletion of food, or the ratio of some essential constituent to the number of cells, might be concerned with retardation or cessation of growth. In any event, the evidence points to the fact that growth is retarded by some factor other than growth toxins. The diminution of toxicity was not due simply to a buffering action of the peptone. The renewal of food supply or some vital constituent remains open.

When the culture was aerated at intervals, the rate of growth was appreciably accelerated and the twenty-four-hour crop

approximately doubled. Following, however, ensued a rapid drop in the aerated culture while the control culture continued a slow increase so that the populations in the two approximated each other in thirty-six to forty-eight hours. Further aeration resulted in unimportant changes. This again might indicate depletion of some vital constituent, but the present knowledge of bacterial requirements and peptone composition stand in the way of proving or disproving this possibility. There is the further

TABLE 1

*Effect of addition of concentrated nourishment to old cultures*

| GROWTH PERIOD | ADDITION OF MEDIUM | CONTROL | PARTIAL REPLACEMENT | CONTROL |
|---|---|---|---|---|
| *hours* | | | | |
| 0 | 2.3* | 2.3* | | |
| 24 | 1,240 | 1,240 | 1,900*† | 1,900* |
| 75 | 700 | 760 | 1,600† | 900 |
| 80 | † | | | |
| 96 | 1,280† | 720 | 1,800† | 700 |
| 125 | 1,220 | 630 | 1,500 | 400 |
| 131 | † | 410 | | |
| 145 | 500 | | | |
| 155 | † | | | |
| 170 | 400† | 112 | | |
| 193 | 510† | | | |
| 216 | 400† | 9 | | |
| 230 | 180 | | | |

* Cells per milliliter (in millions).

† Peptone added.

possibility of some volatile inhibiting substance being removed by the aeration. This likewise is difficult to establish satisfactorily, yet when nitrogen was used instead of air with restoration of the air environment after bubbling no significant increases in populations were noted. There remains for consideration the effect of the aeration on the oxidation-reduction potential of the system and this will be considered in a later paper. A system whereby food could be renewed at any desired rate and the accumulations of wastes prevented might provide a means of evaluating more satisfactorily the significance of the various controlling factors.

The device adopted by Rogers (1930) suggested a means of approaching such conditions. The arrangement shown in the diagram was developed and utilized in further studies. Broth flows from a reservoir through a capillary into a 500 ml., three-necked flash which serves as the culture chamber. An automatic siphon maintains a constant volume in the chamber, discharging

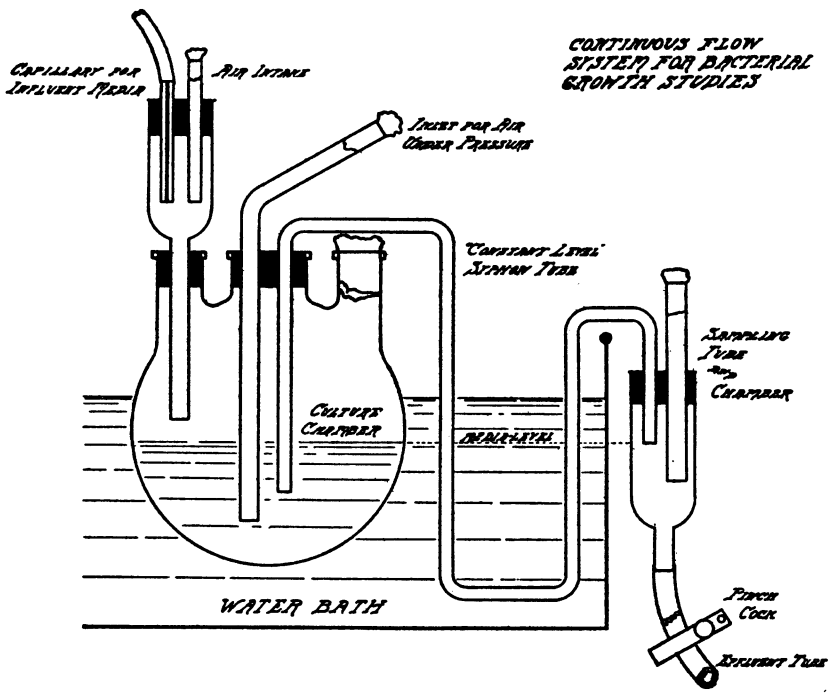


FIG. 1

the overflow into sterile bottles. Rate of flow can be varied from 1.0 to 500 cc. per twenty-four hours by changing the level of the food reservoir. Rate of change in the culture chamber may be controlled by varying the volume of the culture. The organisms are virtually growing in a constant stream of fresh food. Wastes are at least maintained at a significantly lower level than in the ordinary stationary culture. The effect of varying peptone concentrations, of glucose as an additional energy source both in

buffered and unbuffered broths, and of aeration by means of a motor driven stirrer were investigated in this continuous-flow set-up.

The effect of the continuous flow arrangement was very apparent. In a stationary culture the count rose to 189 $\times$ $10^7$ in twenty-four hours and decreased to 140 $\times$ $10^6$ after one hundred sixty-eight hours. In the continuous-flow the count rose to 140 $\times$ $10^7$ in twenty-four hours and was maintained at that level through one hundred ninety-two hours. The population peak attained is approximately the same in both cases. In the constant flow more time is required to reach this peak since organisms

TABLE 2

*Effect of glucose and continuous flow on size and maintenance of population*

| TIME | STATIONARY CULTURE—NO GLUCOSE UNBUFFERED | | CONTINUOUS FLOW NO GLUCOSE UNBUFFERED | | STATIONARY 0.25% GLUCOSE BUFFER | | CONTINUOUS 0.25% GLUCOSE BUFFERED | |
|---|---|---|---|---|---|---|---|---|
| | Cells per milliliter* | pH | Cells per milliliter | pH | Cells per milliliter* | pH | Cells per milliliter* | pH |
| *hours* | | | | | | | | |
| 0 | | 7.2 | | 7.2 | | 7.2 | | 7.2 |
| 24 | 128 | 7.5 | 79 | 6.7 | 174 | 6.4 | 228 | 6.5 |
| 48 | 107 | 8.0 | 115 | 6.9 | 227 | 7.3 | 315 | 6.9 |
| 72 | 63 | 8.2 | 130 | 7.1 | 137 | 7.5 | 370 | 7.1 |
| 96 | 40 | 8.4 | 190 | 7.1 | 83 | 7.6 | 325 | 7.0 |
| 120 | 17 | 8.7 | 190 | 7.1 | 63 | 8.2 | 320 | 7.1 |

* Times 10 million.

are continually being lost to the overflow. That mere lack of food was not the only factor is evidenced by the fact that when the culture was filtered and reseeded a population of 600 million was reached. This observation is of course not new. At the end of one hundred ninety-two hours, glucose was added to the constant flow to give an approximate concentration of 0.25 per cent and in twenty hours the population had jumped to 195 $\times$ $10^7$. A severe drop followed, due probably to the drop in pH to 5.3.

The next experiment indicated the effect of glucose and pH changes more clearly. Observations on 1.0 per cent peptone cultures containing 0.25 per cent glucose buffered with M/50 phos-

phate in both stationary and continuous flow set-ups, in contrast with a stationary peptone culture without glucose, are shown in table 2.

In this experiment as in the others the effect of waste accumulations is apparent. The pH effect is well correlated with maintenance of the population. This is, of course, especially noticeable when the sugar media are unbuffered.

In this experiment, too, there are suggestions that some other factor than toxicity is acting to restrain growth. The effect of food additions makes this evident. That it is not necessarily mere lack of nourishment in form of cell-building constituents may be inferred from the experiment before cited in which old broth was re-inoculated, and also from the effect produced by adding a readily available form of energy such as glucose. When this is added there is not only an increased growth rate, but the final populations are significantly higher, and under conditions where the accumulations of wastes are prevented and both food and energy are continuously renewed this high population level may be maintained indefinitely. The effect of glucose is significantly indicated by comparing two constant flow chambers, one of which carried glucose and the other merely peptone.

## DISCUSSION

The inference may be drawn that growth is restrained first by a change in the ratio of cells to all building material. That food requiring the least amount of energy for reorganization is first utilized. With these compounds exhausted or reduced, constituents requiring more energy for reorganization are utilized, but since more energy is required and less per cell available, the bacterium becomes less efficient and growth is checked. Possibly at this point the effect of the metabolites on the cellular enzymatic activities becomes apparent and the weakened cells die.

The above speculations gain credibility by recalling the studies of Clifton (1933) on "resting bacteria." It was pointed out that while the rate of reduction of ferricyanide increases with concentration of peptone up to concentrations of 2.0 to 3.0 per cent, further increases in concentration bring reductions in the rate.

Likewise it has been noted in the present studies, that higher concentrations than 2.0 per cent peptone fail to increase significantly the numbers of cells. Changes in hydration and viscosity of the substrate probably begin to play an important rôle beyond this point, although data are not available. Clifton further points out that the rate of reduction per cell per minute of ferricyanide in a 1.0 per cent peptone solution is reduced with increasing numbers of cells, and increases as the concentration of the oxidant is increased. Those data may be regarded as the resultant of bacterial activity—the intensity and duration of which will determine the population at any interval.

In the normal growing culture of organisms concentration of cells is continually increasing and concentration of oxidant decreasing. Consequently a decrease in bacterial activity as reflected by numbers of cells is to be expected. Even at this point growth may be again accelerated by the addition of available energy. This cannot be long supplied by peptone alone. More readily available energy in the form of glucose brings about further increase in numbers, continuing until the maximum efficiency for the amount of energy, the type and amount of cell building material is reached. This will logically vary with the species. Increase of cells cannot further occur unless energy is introduced which is more easily available or some other method of increasing enzymatic efficiency is invoked.

## SUMMARY AND CONCLUSIONS

An apparatus for studying growth phenomena in a constant flow is described.

Physical crowding is unimportant in restraining bacterial growth.

The formation of specific growth-inhibiting substances is discounted by the experiments dealing with growth acceleration of cultures in the phase of decline.

The effect of metabolites is a variable, depending on the metabolite and its effect on the enzymatic mechanism of the cell.

Growth will not mount above a certain point even with the

effect of metabolites reduced to a low level. We believe this to be because of the reduction in energy available per cell.

Growth ceases because of changes in the availability of nutrient material and energy demand.

## REFERENCES

GRAHAM-SMITH, G. S. 1920 The behavior of bacteria in fluid cultures. Jour. Hyg., **19**, 133.

HENRICI, A. T. 1928 Morphologie Variation and Rate of Growth of Bacteria. Springfield, C. C. Thomas.

ROGERS, L. A., AND WHITTIER, E. O. 1930 Growth of bacteria in a constant flow of broth. Jour. Bact., **20**, 127.

RAHN, OTTO 1932 Physiology of Bacteria. Philadelphia, P. Blakeston Son and Company.

CLIFTON, C. E. 1933 Factors influencing rate of reduction of potassium ferricyanide by resting bacteria. Proc. Exper. Biol. and Med., **31**, No. 1, 109.

CLIFTON, C. E., CLEARY, J. P., AND BEARD, P. J. Oxidation-reduction potentials, and ferricyanide reducing activities in peptone cultures and suspensions of Escherichia coli. Jour. Bact., 1934, **28**, 541.

CLIFTON, C. E., AND CLEARY, J. P. 1934. Oxidation-reduction potentials and ferricyanide reducing activities in glucose-peptone cultures and suspensions of Escherichia coli. Ibid. 561.