The Effect of pH at Different Temperatures on the Growth of *Bacterium coli* with a Constant Food Supply

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SUMMARY: The effect of pH over the range 5–9 on the growth of *Bacterium coli* with a constant food supply was studied at 20 and 30°. Total and viable counts were made and growth curves constructed. To discover the effects of starvation, sampling was continued after stopping the food supply.

The total count always substantially exceeded the viable. Each growth curve showed an 'initial phase' of varying daily increment in cell numbers merging into a 'steady phase' of roughly constant increment, which continued as long as food was supplied. Low pH slightly shortened the initial phase, low temperature greatly prolonged it. In the early initial phase development was slow at pH 5, but later became exceedingly rapid. Altogether, the conversion of the food supplied into (total) bacterial cells was best effected in conditions of low temperature and low pH, low temperature being the more important. These conditions also favoured high viable counts, and consequently smaller non-viability indices.

During starvation the apparent total counts declined, except at 30° and pH 5, when a steady increase occurred. Higher pH and lower temperature led to faster rates of decline. Viable counts remained approximately constant at pH 5, but otherwise the numbers declined.

Jordan & Jacobs (1944) showed that *Bacterium coli* could be cultivated successfully in an apparatus which permitted accurate control of temperature, pH and aeration, and enabled food to be added at a constant rate, so that the growth was controlled by the food supply. The apparatus was fully described and the results of preliminary experiments reported. After an initial phase, growth was shown to continue at an approximately steady rate so long as food was added. Subsequently, the apparatus was used to investigate the influence of temperature on the growth curves (Jordan & Jacobs, 1947), when it was shown that at pH 7 low temperatures were the most favourable to growth, in that they conduced to the formation of the largest amounts of bacterial cell substance from a given amount of food. Temperature also had a profound influence on the formation of viable cells. The proportion of viable cells formed was greatest at the lowest temperature used (15°), but at 35°, which is close to the temperature ordinarily regarded as the optimum for this organism, roughly half the cells formed were not viable, so that the number of viable cells remained virtually constant while the total cells steadily increased. At lower temperatures the viable count also increased. In view of these interesting findings it was decided to extend the experiments to determine how the cultures would behave under other conditions of pH. The results of this work are reported below.

METHODS AND RESULTS

Apparatus and technique. The apparatus and technique employed were fully described in the first paper of this series, and a brief summary was included in the second (Jordan & Jacobs, 1944, 1947). In the experiments described below the various pH values were all secured by the use of appropriate phosphate mixtures, since it was essential that different ions should not be introduced. Total counts were made by a turbidimetric method.

Experiments performed. Experiments were carried out at pH 5 and 6 at both 20 and 30°, and at pH 9 at 20°, daily estimations being made of the total and viable cells present. The rate of food addition was 0.066 ml./100 sec. of a solution of Difco dehydrated broth, equivalent to an actual addition of 15.2 mg. dehydrated broth/hr. After a time which varied between 14 and 21 days in different experiments the food supply was stopped, but sampling continued, in order that the effects of starvation could be studied. The results are too extensive for detailed presentation in tabular form, so they are shown graphically in Figs. 1-5.

General remarks. The main features of the growth curves at all pH values are similar and resemble those obtained when the effect of varying the temperature at a constant pH of 7 was being investigated (Jordan & Jacobs, 1947). They are, in brief, as follows: (1) The total count was always considerably higher than the viable. (2) An initial phase, in which the daily increments in the numbers of total and viable cells were changing rapidly, was succeeded by a steady phase, characterized by approximately constant increments. (3) There were indications that in the steady phase growth of the cultures had actually occurred in steps. (4) After the food supply was stopped, in most cases the numbers of viable cells and apparent total cells soon began to decrease.

Despite the tendency in the present experiments for the bacterial numbers to increase in steps during the steady phase, it was, as before, convenient to treat the data as if each culture had grown at a constant rate in that phase. The equations of the straight lines which best fit the data have been calculated (Table 2), and the lines drawn in Figs. 1-5 correspond to these. The linear treatment is justified by the smallness of the standard errors of these slopes (ΔT and ΔV respectively for the total and viable cells), but if the stepwise increase was real then the divergences from linearity were systematic and the standard errors have little significance. The slopes of the lines afford a useful assessment of the behaviour of the cultures as a whole, and enable a comparison to be made of the effects of the different conditions of pH and temperature. Also, as the sigmoid curve of growth during the initial phase should flow smoothly into the straight line of the steady phase, the latter has assisted in fixing an approximate duration for the initial phase. The end of that phase being coincident with the beginning of the steady phase, the equations representing the steady phase have been given in the appropriate form. The starvation phase has also been treated as if the cell numbers changed linearly with time, and in the figures smooth curves have been drawn to link the straight lines.

Fig. 1. Growth curves for *Bact. coli* on a constant food supply of 15.2 mg. Difco broth/hr. at 30° and pH 5.0. The food supply was stopped shortly before taking sample marked by arrows.

Fig. 2. Growth curves for *Bact. coli* on a constant food supply of 15.2 mg. Difco broth/hr. at 30° and pH 6.0. The food supply was stopped shortly before taking sample marked by arrows.

Fig. 3. Growth curves for *Bact. coli* on a constant food supply of 15.2 mg. Difco broth/hr. at 20° and pH 5.0. The food supply was stopped shortly before taking sample marked by arrows.

Fig. 4. Growth curves for *Bact. coli* on a constant food supply of 15.2 mg. Difco broth/hr. at 20° and pH 6.0. The food supply was stopped shortly before taking sample marked by arrows.

The initial phase

It is highly probable that food, which was being added continually, at first accumulated, because the 300–400 cells/ml. of the inoculum were unable to use it as fast as it was supplied. Later, this accumulation would be used more or less rapidly according to conditions, until it was exhausted and the culture entirely dependent on the regular additions of food. The establishment of that state is held to mark the end of the initial phase.

Fig. 5. Growth curves for *Bact. coli* on a constant food supply of 15.2 mg. Difco broth/hr. at 20° and pH 9.0. The food supply was stopped shortly before taking sample marked by arrows.

Clearly, at both temperatures the commencement of development of the cultures at pH 5 was considerably delayed, but when growth did start it was exceedingly rapid, due presumably to the accumulation of a relatively large amount of food during the period of delay. This delay was longer at 20 than at 30°, and this was also evident at pH 6.

In Table 1 the duration of the initial phase, the total and viable cell populations at its termination, and the ratio of these values for each experiment, are given, together with the final cell yield in relation to the duration of the initial phase, i.e. in relation to the total food added during that phase. To complete the picture, data from the earlier experiments at pH 7 are included in this and subsequent Tables. The initial phase was longer at the lower temperature and the yield of cells greater both absolutely and relative to the phase length. This agrees with previous findings at pH 7 (Jordan & Jacobs, 1947). The duration of the initial phase tended to be shorter at the lower pH values, but the effect was slight, and it is noteworthy that the long delay in development at

pH 5 had a barely perceptible influence on the duration of the initial phase. Also, the total and viable cell populations at the end of the phase varied but slightly, being on the whole smaller at the lower pH values. In consequence, the cell yields were, with one exception, directly proportional to the phase length. They depended directly on the amount of food added during the phase, and variations of pH within wide limits had no appreciable overall effect on the conversion of food into cells. The exception is the case of 20° and pH 9, where, despite a normal yield of total cells, that of viable cells was depressed, indicating a tendency for a larger proportion of the cells formed to be non-viable or to lose their viability readily.

Table 1. *The duration of the initial phase and the yield of cells under various conditions of pH and temperature*

Temp. (° C.)	pH	Duration of initial phase (days)	Yield of cells at end of initial phase (millions/ml.)		Ratio of total to viable count	Mean rate of cell formation (millions/ml./day)	
			Total	Viable		Total	Viable
20	5.0	7	1280	765	1.67	183	109
	6.0	7	1290	820	1.57	184	117
	7.0	8	1480	880	1.68	185	110
	9.0	8	1530	655	2.34	191	82
30	5.0	5	750	390	1.93	150	78
	6.0	4.5	650	315	2.06	144	70
	7.0	6	945	445	2.12	159	74

The constancy of the ratio of total to viable cells at a given temperature follows from the absence of any marked effect of pH on the cell yields. But whereas the ratios at 30° are all close to 2.0, with a slight tendency for the value to fall with increasing acidity, at 20° the values (apart from the exception noted) are lower and have a mean of 1.64 (Table 1). According to this evidence the value of the ratio would appear to be characteristic of the temperature and independent of the pH within certain limits.

The steady phase

The effect of pH on the behaviour of the cultures during the steady phase is clearly revealed by the values of the slopes of the calculated lines of regression of total and viable count on time (ΔT and ΔV respectively), which are given in Table 2. As anticipated from experiments at pH 7 previously reported, ΔT and ΔV were both larger at 20 than at 30° at pH 5 and 6. The effect of variation in pH on ΔT was slight in comparison with that of temperature, but quite definite, and clearly at both temperatures a low pH tended to enhance the yield of cell substance from a given amount of food. This effect was not apparent at the end of the initial phase, probably in part because of the shortness of that phase and in part because of the complicated conditions of food concentration then existing. Above neutrality an increase in pH had very little effect on ΔT , but below neutrality its increase with falling pH was approximately linear over the range studied. The effect of pH variation on

ΔV was generally similar to that on ΔT but rather more marked, and the alkaline reaction had a distinctly adverse influence.

The ratio $\Delta T_{20}/\Delta T_{30}$ is in the nature of a temperature coefficient, and it is noteworthy that its magnitude is practically independent of pH, the actual values being 1.87, 1.80 and 1.78 at pH 5, 6 and 7 respectively. The ratio $\Delta V_{20}/\Delta V_{30}$ is less constant, although two of the three values are very similar. They are respectively 2.11, 1.64 and 2.07. With increasing acidity, at both temperatures ΔV was actually enhanced in relation to ΔT . This aspect is best

Table 2. *The effect of pH on the linear relation between numbers of total and viable cells and time in the steady phase, and on the non-viability index*

Temp. (° C.)	pH	Equation of regression of total count on time $T = T_s + \Delta T(t - t_s)^*$	Standard error of ΔT	Equation of regression of viable count on time $V = V_s + \Delta V(t - t_s)^*$	Standard error of ΔV	Non- viability index†
20	5.0	$T = 1280 + 141.2(t - 7)$	± 5.27	$V = 765 + 75.3(t - 7)$	± 5.71	0.233
	6.0	$1290 + 128.6(t - 7)$	± 4.36	$820 + 50.2(t - 7)$	± 1.68	0.305
	7.0	$1480 + 118.9(t - 8)$	± 3.86	$880 + 50.6(t - 8)$	± 2.23	0.287
	9.0	$1530 + 115.2(t - 8)$	± 7.05	$655 + 33.8(t - 8)$	± 1.31	0.354
30	5.0	$750 + 75.7(t - 5)$	± 7.81	$390 + 35.7(t - 5)$	± 3.70	0.264
	6.0	$650 + 71.4(t - 4.5)$	± 2.21	$315 + 30.7(t - 4.5)$	± 1.14	0.285
	7.0	$945 + 66.8(t - 6)$	± 2.41	$445 + 24.4(t - 6)$	± 3.28	0.318

* T_s = total cell count in millions/ml. at start of steady phase.

t = time in days.

t_s = time at which steady phase began.

ΔT = rate of increase in total count in millions/ml./day.

V_s = viable count in millions/ml. at start of steady phase.

ΔV = rate of increase in viable count in millions/ml./day.

† non-viability index = $\frac{1}{2}(1 - \Delta V/\Delta T)$.

expressed in terms of the non-viability index (n.v.i.), previously defined as the proportion of the cells formed in a generation which is non-viable (Jordan & Jacobs, 1944, 1947). This index is obtained by calculating the quantity $\frac{1}{2}(1 - \Delta V/\Delta T)$ and its values are also given in Table 2. With one irregularity at 20° and pH 6, the n.v.i. fell with diminishing pH, and thus the conclusion is reached that for the maximum yields of cell substance and of viable cells from a given amount of food, a low temperature and acid conditions are most favourable. This was not reflected in the yields of cells at the end of the initial phase, probably because the effect of acidity, though definite, was relatively slight and the time was too short for an observable effect to appear, except at pH 9. At this alkaline reaction the n.v.i. had the highest value of those recorded in the present series of experiments.

The starvation phase

Assessment on a simple basis of the behaviour of the cultures during starvation is difficult because the changes in turbidity and numbers of viable cells were not always regular nor consistently in one direction. There was a suggestion in some of the growth and death curves of a step-wise change, but the general trends are revealed if the starvation phase is treated in the same way as the steady phase, i.e. as if regular daily increments or decrements had

occurred. The behaviour of each culture can then be summarized in terms of the slopes of the calculated lines of regression of cell numbers on time (Table 3). As a result of adopting this procedure it becomes evident from the figures that in all experiments except one, the apparent total cell count dropped suddenly immediately after the food supply was stopped. This is attributed to shrinkage of the cells in the changed environment, and implies that the factor for converting turbidities to cell numbers had altered.

Table 3. *The average daily changes in total cell count (ΔT) and viable count (ΔV) during starvation, at various values of pH and temperature*

Temp. (° C.)	pH	ΔT (millions/ml./day)	ΔV (millions/ml./day)
20	5.0	-17.9 ± 10.9	$+ 2.1 \pm 2.2$
	6.0	-22.6 ± 3.4	-11.2 ± 2.6
	9.0	-40.2 ± 3.5	-40.3 ± 4.0
30	5.0	$+21.2 \pm 6.1$	$+ 4.3 \pm 6.1$
	6.0	-13.3 ± 1.8	-33.8 ± 1.9
	7.0	-31.5 ± 3.6	-22.8 ± 3.5

Apart from the case of 30° and pH 5, the apparent total cell count decreased during starvation, presumably as a result of autolytic processes leading to loss of opacity and disappearance of cells. These changes evidently became less rapid as acidity increased, and at the higher temperature. The latter finding agrees with results obtained at pH 7. Higher temperature may favour cell persistence by enhancing food storage by the cells, since from a given amount of food fewer cells are formed as the temperature is raised. The case of 30° and pH 5, where the total count continued to rise during starvation, is not really exceptional, since it accords with the general hypothesis that autolysis is reduced by acidity and higher temperature, but it would seem that considerable food storage must have occurred during the preceding phases to provide for the continued growth. This growth was balanced by death, since the viable count remained virtually constant, as it also did at 20°, pH 5. At pH 6, at both temperatures, the viable cell numbers continued to rise for a short time after the food was stopped, before a decline commenced. In these cases, therefore, the values of ΔV in Table 3 refer to the period following the rise. It appears that whereas the rate of fall was slow at 20°, a marked decline occurred at 30°, a result in accordance with that previously obtained at pH 7. At the only alkaline reaction employed the rate of decline was the highest of all. The picture is, on the whole, consistent, and it appears that the cells of these starved cultures survived best under acid conditions.

DISCUSSION

When the optimum pH and the limits of reaction for the growth of a bacterium are determined by the usual method, the presence of growth and its amount are judged by the degree of turbidity developed, so that total and not viable growth is the criterion by which the effect of pH is measured. Three factors

are concerned in the growth of the cultures, namely, the duration of the lag phase, the rate of growth in the logarithmic phase, and the maximum population which the medium can support. Ordinarily no differentiation is made between these factors, but it would obviously be possible to determine the effect of changes in pH on all of them and to establish an optimum for each. These need not be identical.

Hinshelwood and his colleagues have established that the effect of pH on the growth of *Bact. lactis aerogenes* is exerted wholly on the level of the maximum population, there being no influence at all on the lag phase or the rate of growth (Hinshelwood, 1946). This result, if generally true, might lead to a definition of optimum pH as the value conducing to the maximum total cell population that a given medium can support. The growth curves of *Bact. coli* cultures supplied with food at a constant rate are, as shown above, very different from those grown under normal conditions of excess initial food supply. For instance, there is no evidence of the existence of a maximum population, and under both acid and alkaline conditions growth continued unchecked as long as food was supplied. The same was true of the viable cell numbers, and for these cultures the definition of optimum pH based on a maximum cell population is obviously meaningless. Constant growth rates, expressed in terms of the rates of increase in total and viable cells, did, however, exist, and they rose as pH was reduced. Actually, the maximum rates may not have been reached even at pH 5, the lowest value tested, but it seems probable that an optimum pH could be defined for these cultures as the value at which these rates were highest.

Cultures grown in the normal way with an initial excess of food may react to pH differently from those developing on a restricted but regular food supply. Shimwell (1935), working with *Lactobacillus pastorianus*, used acid production as the measure of growth. This probably depends on total rather than viable cell numbers, and this author also found that the growth rate was independent of pH. However, he was able to show that the lag phase increased as the pH was reduced. The results of the experiments described above suggest, although do not prove, that the lag phase of *Bact. coli* was increased at pH 5. Evidently the effect of pH on bacterial cultures is no simple one exerted invariably on a single phase of growth, but may vary from organism to organism and also with the method of cultivation.

It is clear that acidity, at least down to pH 5, increasingly favoured the growth of *Bact. coli* under these conditions of constant food supply, and also led to a greater viability among the cells formed. This behaviour is in striking contrast to the sensitivity to acidity of many of the enzymes of *Bact. coli*, as demonstrated by Gale & Epps (1942). These authors also showed that *Bact. coli* would grow in a casein digest between pH 4.5 and 9, and responded to changes in the external pH by alterations in the enzyme content of the cells, such that the external changes were counteracted and certain essential activities maintained at a constant level. No doubt the cells of cultures with constant food supply, when grown under acid or alkaline conditions, also responded in this manner, but it would be expected that towards the limits of the pH range

the cells would be functioning with a somewhat reduced efficiency. Actually, near the lower limit of the range the cells were functioning more efficiently than at higher pH values, and this suggests that some other factor, as yet undiscovered, was predominant. The increased efficiency was shown not only in the higher growth rate and viability, but also apparently in the storage of reserve food, by virtue of which cell multiplication was able to continue for a considerable time after cessation of the food supply.

Previous results (Jordan & Jacobs, 1947) had shown that at pH 7 the ratio of total to viable cells at the close of the initial phase approximated to the value of 2.0 over a wide range of temperature. In the work reported above this ratio was also close to 2.0 at pH 6 and 5 when the temperature was 30°. However, at 20° the ratio at these pH values was lower (about 1.7), whereas at pH 9 it was higher (2.3). In the earlier work a value of 1.68 was obtained at 20° and pH 7 and was then regarded as a chance variation from the true value of 2.0, but since other similar values have now been obtained at that temperature under more acid conditions, and a value higher than 2.0 at an alkaline reaction, it is now considered that the value of 2.0 may be more susceptible to change with alteration of environmental conditions than was formerly thought to be the case.

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