The Influence of pH Value and Aeration on the Growth of *Aerobacter aerogenes* and *Bacterium coli* in defined Media

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SUMMARY: The total amount of growth of *Aerobacter aerogenes* and *Bacterium coli* in glucose ammonium salt media which provided substrates in excess was dependent on the initial pH value in aerated and unaerated cultures. Total crops in aerated cultures were always higher for any particular initial pH value, and for cultures inoculated at pH values less than 7.0 the pH fell to lower values in aerated than in unaerated media. Growth of a culture of *A. aerogenes* in its (unaerated) stationary phase resumed on aeration; when the pH value at inoculation was 5.0–6.0, the pH continued to fall; when the pH value at inoculation was 6.1–7.0, the pH rose slightly. Growth of similar cultures of *Bact. coli* lagged when aerated; pyruvate concentrations immediately increased and pH values fell when glucose was in excess but not when it was limiting for growth. The concentration of formic acid produced by fully grown cultures was sufficient to abolish growth in fresh media below pH 5.25 but not at pH 7.0. These observations are discussed in relation to views on the influence of aeration on growth.

Relationships between total growth and initial pH value of citrate media were established for aerated and unaerated cultures of *A. aerogenes*. Growth in unaerated media ceased at pH values slightly higher than those at inoculation, leaving some citrate unconsumed. Aeration then caused growth resumption accompanied by consumption of citrate and a further increase in alkalinity.

Although the influence of pH value on bacterial growth has been frequently observed, Stephenson (1949) stated that much published work is vitiated because it takes into account only the initial pH value. Gale & Epps (1942) followed pH changes during growth of *Bacterium coli* in complex media. Lodge & Hinshelwood (1939) found that stationary populations of *Aerobacter aerogenes* in aerated glucose ammonium salt medium were profoundly influenced by the initial pH value of the medium. We have measured pH changes during growth in various defined media with and without aeration. Ravin (1952) suggested that growth cessation in unaerated media is due solely to development of adverse pH values. The increased bacterial crop on aeration may be due to a more favourable pH value which is achieved when accumulated organic acids are consumed. We have tested this speculation by measuring pH changes when unaerated cultures in their stationary phase were aerated.

METHODS

The organisms used throughout this work were *A. aerogenes* NCTC 418 and *Bact. coli* NCTC 5928. The media used contained KH_2PO_4 , 5.4 g.; $(\text{NH}_4)_2\text{SO}_4$, 1.2 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4 g., made to 1 l. with glass-distilled water and, unless otherwise stated, the required carbon sources were added to give concentrations of 12 g. glucose and 35.3 g. Na citrate dihydrate/l. The pH value was adjusted to that required by the addition of 5N-NaOH or 5N-HCl. Cultivation was at 37° in either 6 × 1 in. or 8 × 1½ in. Pyrex tubes. Cultures were aerated by passage of a gentle stream of sterile air. Methods of obtaining growth curves and precautions necessary to ensure reproducibility have already been described (Dagley, Dawes & Morrison, 1950*a*). Samples for pH and other determinations were obtained by centrifugation of cultures. Marconi type TF889 and Muirhead type D417A pH meters with glass electrodes were used for pH measurement. Titration curves for the media were prepared against 5N-NaOH and 5N-HCl to enable pH changes to be expressed as amount of acid or alkali produced per cell.

Pyruvic acid was determined by the toluene extraction method of Friedemann & Haugen (1943) and citric acid by the method of Perlman, Lardy & Johnson (1944). Glucose concentrations were determined by the Somogyi (1937) method and ammonia by nesslerization. Formic acid was determined by the A.O.A.C. (1945) method and the acetic acid by the difference between the total volatile acid concentration, obtained by steam distillation and titration with 0.1N-NaOH, and the formic acid concentration. Acetoin was determined by the method of Happold & Spencer (1952*a*).

RESULTS

Relationship between total growth and initial pH of glucose medium

Growth of cultures containing excess substrates (Dagley, Dawes & Morrison, 1951) at a series of initial pH values was followed turbidimetrically until growth ceased. In agreement with the results of Lodge & Hinshelwood (1939) we found that the crop was considerably affected but that the mean generation time was not. Final bacterial populations for aerated and unaerated cultures are shown in Fig. 1. For both organisms over the whole pH range higher final populations were obtained in aerated than in unaerated media. When the initial pH value was <6, *A. aerogenes* grew to higher populations than *Bact. coli* in both aerated and unaerated cultures. From the titration curve for the medium and measurements of pH values at the cessation of growth, the amount of acid formed was calculated. The amount of acid accumulated per million cells is plotted in Fig. 1; for *A. aerogenes* this value falls sharply to a minimum at pH 5.8. Determinations of acetoin in growing cultures showed a rapid increase in concentration when the pH fell below 6.0; the pH optimum for the reaction is given as 5.6–6.0 (Silverman & Werkman, 1941; Happold & Spencer, 1952*b*; Juni, 1952) and this minimum in the curve of acid production for *A. aerogenes* may probably be attributed

to acetoin formation. For both organisms acid production in unaerated conditions was higher and in all cases maxima were given at *c.* pH 7.5.

Effect of aeration on pH value of glucose medium

Measurements of pH value within 1 hr. of growth cessation (Table 1) showed that with initial pH values <7 aerated cultures continued to grow when the pH had fallen to a value which inhibited growth in unaerated media. From these results it appeared that growth with aeration was less sensitive to high acidity than semi-anaerobic growth. We therefore investigated changes

Fig. 1. Relationship between initial pH of glucose ammonium salt medium, total growth and acid accumulation, for *A. aerogenes* and *Bact. coli* in aerated and unaerated cultures. Growth: aerated, ○; unaerated, ●. Acid accumulation, culture: aerated, +; unaerated, ×.

in pH value and bacterial population when cultures which had ceased to grow in unaerated conditions were aerated. When the initial pH value of an *A. aerogenes* culture was <6.1, the pH value continued to fall on aeration, whilst a rise in pH value occurred when the initial pH value was between 6.1 and 7.0. For this organism an increase in cell population occurred on aeration at all pH values investigated. Representative results for three pH values are shown in Fig. 2.

Table 1. *Initial and final pH values of Bact. coli and A. aerogenes cultures grown with and without aeration in glucose ammonium salt media*

Initial	pH values						
	5.1	5.5	6.0	6.4	7.1	7.6	8.2
<i>Bact. coli</i>							
Final (not aerated)	4.4	4.6	4.7	5.3	6.0	6.5	7.0
Final (aerated)	4.1	4.1	4.6	4.9	5.8	6.3	6.7
<i>A. aerogenes</i>							
Final (not aerated)	4.1	4.4	5.0	5.4	5.7	5.9	6.8
Final (aerated)	3.8	3.8	3.8	4.7	5.7	6.0	6.9

The initial pH value profoundly influences the balance of end products in bacterial fermentations (Tikka, 1935). We considered it probable that when the pH value increased during aeration, organic acids were being consumed in growth at a rate more than sufficient to counterbalance production of acid by resumed glycolysis, and that in those cases where the pH value fell, glycolysis exceeded acid consumption. In Fig. 2 it is seen that growth did

Fig. 2. Changes in pH during growth of *A. aerogenes* in glucose ammonium salt medium, at three different initial pH values, to the unaerated stationary phase followed by aeration and further growth. Growth, ○; pH, ●. Arrows indicate start of aeration.

not resume immediately on aeration; Lwoff & Monod (1947) reported prolonged lag periods for *Bact. coli* in aerated media. Such delays in growth of our strain of *Bact. coli* were usually of longer duration than those for *A. aerogenes* and permitted us to study the resumption of glycolysis in the absence of growth. The relation between stationary populations and glucose concentrations in the present medium has been reported previously (Dagley *et al.* 1951; Ravin, 1952) and enabled us to measure pyruvate concentrations and pH values for cultures of *Bact. coli* containing amounts of glucose less than, and in excess of, full growth requirements (Fig. 3). In no case was there an increase in pH value on aeration, and pyruvate concentration increased immediately in those cultures where excess glucose remained, but not in those from which glucose was exhausted.

It is seen in Table 1 that growth may cease at a pH value which permits growth to proceed in a fresh medium; e.g. an unaerated *A. aerogenes*, initially at pH 7.2, ceased to grow at pH 5.8, whilst a culture initially at pH 5.8 grew until the pH fell below 5. This suggests that products of metabolism became increasingly toxic as the pH value decreased. Both these organisms produce formic acid, and Gale & Epps (1942) showed that 0.2% (w/v) sodium formate stopped growth of *Bact. coli* below pH 6.2. Determinations of formic acid in filtrates from fully-grown cultures of *A. aerogenes* and *Bact. coli* showed the

presence of 470 and 530 mg. formic acid/l., respectively. The effect of these concentrations of formic acid on growth of these organisms in the range pH 5.0–6.0 is shown in Table 2. Formic acid is increasingly toxic as the pH value decreases, and growth is completely inhibited at pH 5.25 and 5.0 for *Bact. coli* and *A. aerogenes* respectively.

Fig. 3. Changes in pH and pyruvic acid concentration during un-aerated growth and subsequent aeration of stationary phase cultures of *Bact. coli* in glucose ammonium salt media at three different initial pH values. Glucose limiting growth: growth, □; pH, ■; pyruvate concentration, ×. Glucose in excess of growth requirements: growth, ○; pH, ●; pyruvate concentration, +. Arrows indicate start of aeration.

Table 2. Effect of formic acid concentrations found in fully grown cultures of *Bact. coli* and *A. aerogenes* in un-aerated glucose ammonium salt medium on the growth in similar fresh media over the pH range 5.0–6.0

Initial pH value	<i>Bact. coli</i>		<i>A. aerogenes</i>	
	Formate (530 mg./l.) present	No formate	Formate (470 mg./l.) present	No formate
	Stationary populations of bacteria in millions/ml.			
5.0	0	50	0	160
5.25	0	80	75	200
5.50	20	100	350	310
5.75	90	130	460	370
6.0	160	200	490	410

Relation between total growth of Aerobacter aerogenes and initial pH value of citrate medium

Citric acid as source of carbon for growth was studied for two reasons:

- (i) the pH value increases during growth on citrate in contrast to glucose,
- (ii) *A. aerogenes* grows well both aerobically and anaerobically in this medium.

Final bacterial populations were measured in unaerated media at various concentrations of citrate and initial pH value of 7.0. They increased linearly with concentration up to 6×10^{-2} M-citrate, at which concentration a population of 950×10^6 bacteria/ml. was supported. Higher concentrations of citrate produced no increase in crop. With aerated media at initial pH value 7.0 the linear relationship ceased at 3.6×10^{-2} M-citrate, with a population of 1220×10^6 bacteria/ml. For *A. aerogenes* the following comparison may be made of the substrate concentrations required to support a population of 10^6 bacteria/ml.: with aeration, glucose 0.66×10^{-5} M citric acid 2.80×10^{-5} M; without aeration, glucose 1.66×10^{-5} M citric acid 6.95×10^{-5} M.

Fig. 4. Relationship between initial pH of citrate-ammonium salt medium and total growth of *A. aerogenes* in unaerated and aerated media. Growth: aerated, ○; unaerated, □.

Measurements of final populations in aerated and unaerated media containing citrate in excess of growth requirements (35.3 g./l.) were made over a range of initial pH values (Fig. 4). For both aerated and unaerated citrate media there was a sharp decrease in the stationary population when the initial pH values were between 7.0 and 7.5. With initial pH values between 5.0 and 7.0 there was a contrast between unaerated and aerated cultures; for the unaerated cultures there was a decrease of population with decrease of initial pH value and for the aerated cultures an increase. Thus at low initial pH values the increase in crop due to aeration was even greater for citrate than for glucose. Table 3 records pH measurements for cultures in their stationary phase after growth with and without aeration for different initial pH values. In all cases there was an increase in pH value which was

most pronounced in aerated cultures. Changes in pH value of unaerated cultures were so small that cessation of growth could not be attributed solely to this factor. When unaerated cultures at initial pH value 6.85 were aerated in their stationary phase growth resumed and the pH value increased (Table 4). Simultaneous determinations of citrate indicated that citrate decomposition was also resumed on aeration.

Table 3. *Initial and final pH values of A. aerogenes cultures grown with and without aeration in citrate ammonium salt media*

	pH values				
Initial	5.1	5.8	6.3	6.6	7.0
Final (not aerated)	5.3	5.9	6.5	6.8	7.0
Final (aerated)	5.3	6.5	7.6	8.2	7.8

Table 4. *Changes in pH value and citrate concentration when A. aerogenes cultures are grown without aeration to their stationary phase, followed by aeration with resultant growth resumption to their aerated stationary phase*

Time of determination of pH value	pH value		Citrate concentration (mg./ml.)	
	<i>a</i>	<i>b</i>	<i>a</i>	<i>b</i>
At inoculation	6.85	6.85	15.30	15.30
At stationary phase not aerated	7.75	7.60	5.42	5.20
At stationary phase after aeration	8.90	8.80	3.14	2.86

a = 25 ml. culture; *b* = 100 ml. culture.

Table 5. *Effect of acetic acid concentration found in stationary phase unaerated citrate ammonium salt cultures of A. aerogenes on the growth in similar fresh media at various pH values*

	Initial pH				
	6.2	6.4	6.6	6.8	7.1
Stationary populations of bacteria in millions/ml.					
Acetic acid (3.46 g./l.) present	5	13	128	380	600
Acetic acid absent	545	575	725	910	910

Since changes in pH value alone did not account for growth cessation in unaerated media containing excess citrate we investigated the possibility of the formation of toxic products. In the mechanisms proposed by Deffner (1938) and by Brewer & Werkman (1939) citrate is split initially, in anaerobic conditions, to oxaloacetic and acetic acids. Although the oxaloacetic acid inhibits reactions of importance in bacterial metabolism (Stone & Wilson, 1952; Pardee & Potter, 1948) additions of oxaloacetate up to 80 mg./l. increased both rate of growth and final population. The ability of oxaloacetate to promote growth on addition to defined media has been reported previously (Ajl & Werkman, 1948; Dagley, Dawes & Morrison, 1950*b*). Determinations of acetic and formic acids in fully grown unaerated cultures (initial pH 7.0)

gave values of 3.46 and 0.91 g./l., respectively. The addition of this concentration of acetic acid to unaerated cultures inhibited growth in the pH range 6.2–7.1; the effect was more pronounced at low pH values (Table 5).

DISCUSSION

Cessation of growth of *Bact. coli* and *A. aerogenes* in glucose ammonium salt media when nutrients remain unconsumed was attributed to failure of hydrogen transfer from reduced diphosphopyridine nucleotide (DPN) to metabolites (Dagley *et al.* 1951). Production of toxic products was not considered responsible for the end of growth since sterile filtrates from such cultures promote growth (Dagley *et al.* 1950*b*). Such filtrates, however, were tested at pH values which were optimal for growth. The present work shows that formic acid, the toxicity of which increases rapidly as the pH value decreases, is produced in quantities sufficient to inhibit growth at the pH values reached in unaerated media. Inhibition of growth by other end products of metabolism may also increase as acidity develops, and an explanation is afforded of the observation that cultures may cease to grow at pH values which permit growth in fresh media. Ravin (1952) showed that increase of buffer capacity led to increased stationary populations in unaerated cultures and attributed stoppage of growth to the development of adverse pH values, and the resumption of growth on aeration to consumption of accumulated organic acids. We showed, however, that for certain initial pH values acidity continued to develop although growth resumed on aeration. Further, in the present and previous work (Dagley *et al.* 1951), estimations of pyruvate indicated that glycolysis was resumed on aeration, and it seems unlikely that this is without significance for growth.

The effect of acidity on the growth of *A. aerogenes* and *Bact. coli* was more pronounced with unaerated than with aerated cultures. Perhaps hydrogen transfer from reduced DPN to metabolites is inhibited at pH values which permit hydrogen transfer to oxygen. Fowler (1951) showed, however, that anaerobic utilization of glucose by *Bact. coli* appears to be effected by an adaptive enzyme which does not function under aerobic conditions; inhibition by toxic products at low pH values may therefore occur at this point.

The factors which cause growth cessation in anaerobic citrate cultures cannot at present be completely evaluated. Although conditions inside the cell may differ greatly from those outside, measurement of changes of pH value in the external fluid do not support the view that the development of adverse pH values causes growth to cease and the resumption of growth on aeration certainly cannot be attributed to consumption of organic acids formed as fermentation products. Although we showed that acetic acid was produced anaerobically in amounts sufficient to cause inhibition, growth was not abolished at pH 7.

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REFERENCES

- AJL, S. J. & WERKMAN, C. H. (1948). Replacement of CO₂ in heterotrophic metabolism. *Arch. Biochem.* **19**, 483.
- A.O.A.C. (1945). Method described in *Official and Tentative Methods of Analysis of the Association of Official Agricultural Chemists*, p. 535, 6th edn. Washington.
- BREWER, C. R. & WERKMAN, C. H. (1939). The anaerobic dissimilation of citric acid by *Aerobacter indologenes*. *Enzymologia*, **6**, 273.
- DAGLEY, S., DAWES, E. A. & MORRISON, G. A. (1950a). Inhibition of growth of *Aerobacter aerogenes*: the mode of action of phenols, alcohols, acetone and ethyl acetate. *J. Bact.* **60**, 369.
- DAGLEY, S., DAWES, E. A. & MORRISON, G. A. (1950b). Factors influencing the early phases of growth of *Aerobacter aerogenes*. *J. gen. Microbiol.* **4**, 437.
- DAGLEY, S., DAWES, E. A. & MORRISON, G. A. (1951). The effect of aeration on the growth of *Aerobacter aerogenes* and *Escherichia coli*, with reference to the Pasteur mechanism. *J. Bact.* **61**, 433.
- DEFFNER, M. (1938). Die anaerobe Vergärung der Citronensäure durch Bakterien. *Liebigs Ann.* **536**, 44.
- FOWLER, C. B. (1951). The relationship between fermentation and enzymatic adaptation. *Biochim. biophys. Acta*, **7**, 563.
- FRIEDEMANN, T. E. & HAUGEN, G. E. (1943). Pyruvic acid. II. The determination of keto acids in blood and urine. *J. biol. Chem.* **147**, 415.
- GALE, E. F. & EPPS, H. M. R. (1942). The effect of the pH of the medium during growth on the enzymic activities of bacteria (*Escherichia coli* and *Micrococcus lysodeikticus*) and the biological significance of the changes produced. *Biochem. J.* **36**, 600.
- HAPPOLD, F. C. & SPENCER, C. P. (1952a). The bacterial formation of acetylmethylcarbinol and 2 : 3-buteneglycol. *Biochim. biophys. Acta*, **8**, 18.
- HAPPOLD, F. C. & SPENCER, C. P. (1952b). The enzymic formation of acetylmethylcarbinol and related compounds. *Biochim. biophys. Acta*, **8**, 543.
- JUNI, E. (1952). Mechanisms of formation of acetoin by bacteria. *J. biol. Chem.* **195**, 715.
- LODGE, R. M. & HINSHELWOOD, C. N. (1939). Physicochemical aspects of bacterial growth. Part IV. Conditions determining stationary populations and growth rates of *Bact. lactis aerogenes* in synthetic media. *J. chem. Soc.* p. 1683.
- LWOFF, A. & MONOD, J. (1947). Essai d'analyse du rôle de l'anhydride carbonique dans la croissance microbienne. *Ann. Inst. Pasteur*, **73**, 323.
- PARDEE, A. B. & POTTER, V. R. (1948). Inhibition of succinic dehydrogenase by oxalacetate. *J. biol. Chem.* **176**, 1085.
- PERLMAN, D., LARDY, H. A. & JOHNSON, M. J. (1944). Determination of citric acid in fermentation media and biological materials. *Industr. Engng. Chem. (Anal. ed.)*, **16**, 515.
- RAVIN, A. W. (1952). Heritable and non-heritable loss of ability by *Aerobacter aerogenes* to grow adaptively on single carbon sources. *J. gen. Microbiol.* **6**, 211.
- SILVERMAN, M. & WERKMAN, C. H. (1941). The formation of acetylmethylcarbinol from pyruvic acid by a bacterial enzyme preparation. *J. biol. Chem.* **138**, 35.
- SOMOGYI, M. (1937). A reagent for the copper-iodometric determination of very small amounts of sugar. *J. biol. Chem.* **117**, 771.
- STEPHENSON, M. (1949). *Bacterial Metabolism*, 3rd ed. p. 171. London: Longmans, Green and Co.
- STONE, R. W. & WILSON, P. W. (1952). The effect of oxalacetate on the oxidation of succinate by *Azotobacter* extracts. *J. Bact.* **63**, 619.
- TIKKA, J. (1935). Über den Mechanismus der Glucose Vergärung durch *B. coli*. *Biochem. Z.* **279**, 264.

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