

# CITRIC ACID METABOLISM OF AEROBACTER AEROGENES

S. DAGLEY AND E. A. DAWES

Department of Biochemistry, University of Leeds, and Department of Biochemistry, University of Glasgow, Great Britain

Received for publication February 26, 1953

*Aerobacter aerogenes* is able to utilize citric acid as sole source of carbon for growth in both aerobic and anaerobic conditions. From an analysis of fermentation liquors Deffner and Franke (1939) and Brewer and Werkman (1939) concluded that citric acid is decomposed anaerobically to oxalacetate and acetate as initial products. Manometric studies by the latter workers, using *Aerobacter indologenes*, showed that oxalacetic, pyruvic, and L-malic acids were fermented readily in addition to citric acid, and they also suggested that the "citrate enzyme" was adaptive rather than constitutive. Ajl and Werkman (1949) observed the effect of bisulfite and other inhibitors on the ability of citrate to replace the CO<sub>2</sub> requirement of *A. aerogenes* and concluded that the aerobic decomposition of citric acid occurs by a different mechanism. Preparation of bacterial extracts which catalyze some of the reactions of the tricarboxylic acid cycle (Korkes *et al.*, 1950; Stone and Wilson, 1952) has established a pathway for citrate oxidation by this mechanism. Krebs (1950) has drawn attention to the possibility that the dissimilation of citrate to oxalacetate and acetate may be a reversal of the synthesis of tricarboxylic acids and accordingly deserves further attention than it has received so far.

For un-aerated cultures of *A. aerogenes* relationships between bacterial crop and nutrient concentration are linear for glucose (Dagley *et al.*, 1951) and citrate (Dagley *et al.*, 1953) up to certain concentrations, beyond which further additions of carbon source produce no increase in crop in the presence of excess nitrogen source. Aeration of such cultures results in large increases in crop and further consumption of the source of carbon. Among the suggestions to account for the effect of aeration on glucose cultures is that of Fowler (1951) attributing fermentation to an unspecified adaptive enzyme, inactivated by oxygen. Since the effect of aeration on growth of cultures utilizing glucose and citrate is similar, it was of additional interest to study further the

citric acid metabolism of *A. aerogenes* for which a definite adaptive enzyme has been postulated in anaerobic conditions.

## EXPERIMENTAL METHODS

The organism used in this work was *Aerobacter aerogenes*, N.C.T.C. strain 418. Growth curves were constructed by the methods described in earlier work (Dagley *et al.*, 1950). For reasons discussed later, the medium from which cells were harvested contained a concentration of citrate limiting for growth and was as follows. Medium A: Na citrate dihydrate, 7 g; KH<sub>2</sub>PO<sub>4</sub>, 5.4 g; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1.2 g; and MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.4 g per liter glass distilled water; pH adjusted to 7.1. The MgSO<sub>4</sub> was added aseptically as a 10 per cent (w/v) solution after sterilization. In certain experiments a higher concentration of citrate was employed (growth medium B), namely 21 g per liter. Citrate-phosphate solution contained Na citrate dihydrate, 21 g; KH<sub>2</sub>PO<sub>4</sub>, 5.4 g; and MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.4 g per liter glass distilled water; pH adjusted to 7.1. For cells grown with aeration a gentle stream of sterile air was passed through the culture; semianaerobic conditions were obtained in round flasks filled to the neck. Experience showed that cells grown in this latter way had similar properties to those grown under strictly anaerobic conditions in a McIntosh and Fildes jar.

Pyruvic and total keto acids were determined by the method of Friedemann and Haugen (1943), and glucose, by the method of Nelson (1944). Pyruvic and  $\alpha$ -ketoglutaric acids were separated as their 2,4 dinitrophenylhydrazones by paper chromatography; confusion of  $\alpha$ -ketoglutarate with oxalacetate was eliminated by treatment of the metabolizing fluids either with aniline citrate or with 0.5 N HCl at 100 C for 15 min. Identification of the 2,4 dinitrophenylhydrazone of  $\alpha$ -ketoglutaric acid was confirmed by plotting absorption spectra of eluates from chromatograms; details of these procedures have been given already (Dagley *et al.*, 1952).

## RESULTS

*Keto acid production.* Cells were harvested from aerated and from unaerated citrate media, washed, adjusted to equal turbidities, and aerated at 37 C with citrate-phosphate solution. Samples were taken at certain time intervals, the bacteria removed by centrifugation and pyruvate, and total keto acid concentrations determined. Figure 1 shows that the initial rate of total keto acid accumulation for cells grown without aeration is approximately fifty times greater than that for cells grown with aeration. Further, pyruvate accounts for the total keto acid production in the former case but only for a small fraction in the

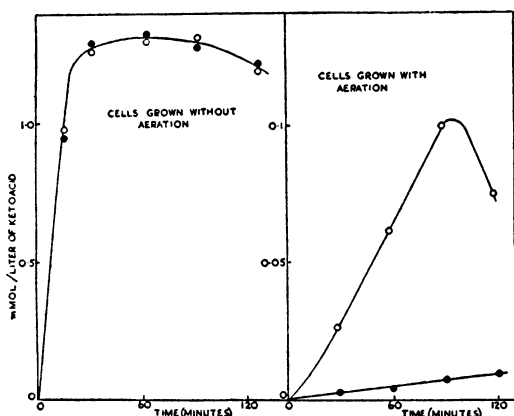


Figure 1. Effect of growth conditions on production of keto acids from citrate by nonproliferating suspensions of *Aerobacter aerogenes*. Keto acid concentrations: total, ○; pyruvic acid, ●. The difference in ordinate scales should be noted.

latter. Analysis of the 2,4 dinitrophenylhydrazones by paper chromatography, as described in the previous section, demonstrated the formation of  $\alpha$ -ketoglutarate in addition to pyruvate by aerated grown cells; no spot corresponding to  $\alpha$ -ketoglutarate was observed for cells grown without aeration. Confirmation of pyruvate as the sole keto acid formed by the latter was obtained as follows. Cells were harvested from 10 liters of anaerobic culture, resuspended in 4 liters of citrate-phosphate solution containing 0.0025 M  $As_2O_3$ , and incubated for 3 hours at 37 C. After Seitz filtration and addition of 300 ml conc HCl and 500 ml 2,4 dinitrophenylhydrazine (0.1 per cent (w/v) in 2 N HCl), the solution was allowed to stand at room temperature overnight. The solution (ca 1.5 liters) obtained after repeated

extraction by ethyl acetate was reextracted with 500 ml 10 per cent (w/v)  $Na_2CO_3$ . The latter on slight acidification with conc  $H_2SO_4$  yielded a yellow precipitate which was filtered and recrystallized from ethyl acetate-ligroin. Yield ca 0.5 g; mp, alone or admixed with authentic 2,4 dinitrophenylhydrazone of pyruvic acid, 213 C. Analysis (Drs. Weiler and Strauss, Oxford): C 40.6, H 3.1, N 21.4.  $C_3H_5O_3N_4$  requires C 40.4, H 3.0, N 20.9 per cent. Experiments similar to those of figure 1 were conducted using a stream of nitrogen in place of air. The concentration of keto acid produced in 30 min by cells grown without aeration was 1,220 and 1,490  $\mu M$  per liter in air and nitrogen, respectively. Cells grown with aeration yielded respectively 100 and 30  $\mu M$  per liter in 60 min. Clearly replacement of air by nitrogen considerably reduces keto acid production by aerobically grown cells but does not similarly affect this capacity in cells grown anaerobically.

*Effect of aeration on adaptation to citrate dissimilation.* From figure 1 it is seen that anaerobic growth in citrate medium confers upon the cells ability to produce pyruvate rapidly from citrate. We have used the following test to measure this ability. A sufficient quantity of cells was harvested, washed, and resuspended in 4 ml citrate-phosphate solution to give a population of  $10^9$  bacteria per ml. After incubation in a 6 by  $\frac{1}{2}$  in tube for 10 min the cells were removed by centrifugation and the keto acid concentration determined to give a quantity  $Q_{KA}$ , expressed as  $\mu M$  per  $10^9$  cells per 10 min.

When inocula for unaerated or strictly anaerobic citrate cultures were taken from broth or glucose-ammonium salt cultures, lag periods occurred. The test described was carried out with cells in this lag phase and no keto acids were detected, but at the onset of the logarithmic phase the cells acquired ability to produce pyruvate. This development of large  $Q_{KA}$  values by cells actively engaged in splitting citrate anaerobically has provided a useful tool for investigating the factors affecting this enzyme system.

Cells from an unaerated citrate culture were harvested, washed, resuspended in citrate growth medium (B) to give a population of ca 250 millions per ml and aerated at 37 C. The  $Q_{KA}$  values of cells withdrawn at suitable time intervals were determined; growth also was followed. Parallel experiments were conducted with cells grown aerobically; after harvesting, washing, and

resuspending in citrate growth medium they were incubated without aeration in 6 by  $\frac{1}{2}$  in tubes. The results of these experiments are shown in figure 2 where it is seen that the former batch of cells progressively lost their high  $Q_{KA}$  values as they grew with aeration, while the second batch exhibited a lag period of 250 min before anaerobic growth occurred and a high  $Q_{KA}$  value was developed rapidly at this time.

Cells harvested from an aerated culture were resuspended in growth medium (B) to give a population of ca  $250 \times 10^6$  cells per ml, and two

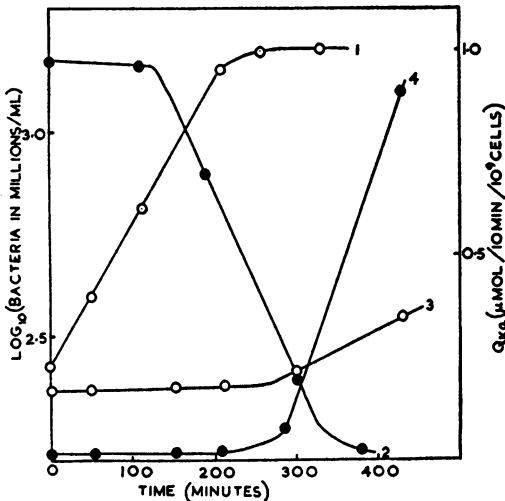


Figure 2. Effect of growth conditions on ability of *Aerobacter aerogenes* to produce keto acids from 0.07 M citrate ( $Q_{KA}$ ). Growth in aerated citrate medium: the inoculum was grown in an un-aerated medium, (1); loss in  $Q_{KA}$  of cells during growth, (2). Growth in un-aerated citrate medium, previous growth aerobic, (3); gain in  $Q_{KA}$  of cells in growth, (4).

50 ml lots were incubated in large boiling tubes at 37 C. One tube was aerated and the other was not; growth was followed in each tube and  $Q_{KA}$  values of samples of cells taken at intervals were determined (figure 3). Growth in the un-aerated medium commenced after a lag period of 50 min, and at that time there was a sudden rise in  $Q_{KA}$  to 1.1. This value was maintained for about 2 hours while the cells were actively dividing, but towards the end of logarithmic growth a sharp fall in  $Q_{KA}$  occurred. The cells which were aerated exhibited no lag phase and their  $Q_{KA}$  value rose only slightly to 0.2.

*Effect of acetate on  $Q_{KA}$  values.* In figure 3 the

decrease in rate of growth towards the end of the logarithmic phase is accompanied by a fall in  $Q_{KA}$ , and this suggests that loss of ability to dissimilate citrate governed the onset of the stationary phase. A relationship between stationary population and citrate concentration for un-aerated cultures providing excess nitrogen

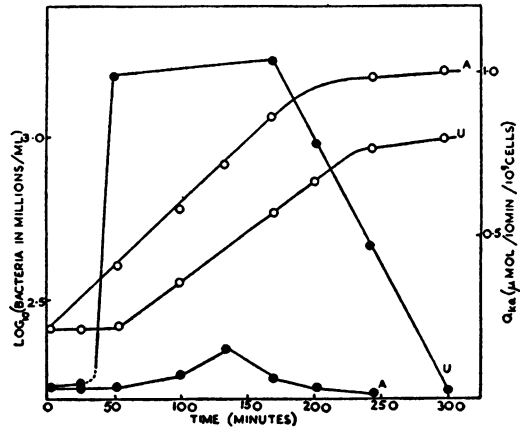


Figure 3. Changes in  $Q_{KA}$  during logarithmic growth. Bacteria were grown in aerated citrate medium and transferred to (A) aerated, (U) un-aerated media. Growth,  $\circ$ ;  $Q_{KA}$ ,  $\bullet$ .

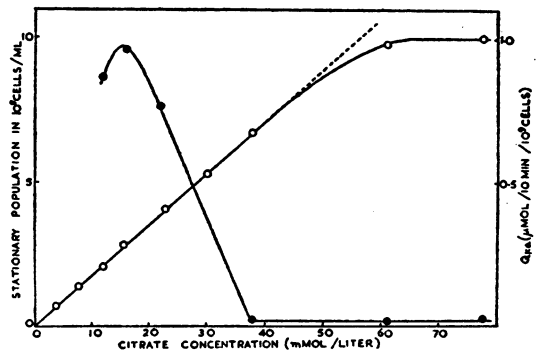


Figure 4. Relationship between stationary population and citrate concentration, shown by open circles. Values of  $Q_{KA}$  for cells harvested from their stationary phases are shown by solid circles.

source (0.2 per cent w/v  $(NH_4)_2SO_4$ ) was obtained by methods previously described (Dagley *et al.*, 1951) and is shown in figure 4. The breakdown in the linear relationship at concentrations higher than  $5.4 \times 10^{-2}$  M citrate indicates that citrate remained unconsumed in the medium at these concentrations, and this has been verified by chemical analysis (Dagley *et al.*, 1953). The

pH of such cultures in the stationary phase is 7.0 to 7.5, suggesting that growth cessation is due solely to the adverse effect of accumulated products of metabolism. For cells grown to their stationary phase in various concentrations of citrate,  $Q_{KA}$  values fell sharply above  $2.7 \times 10^{-2}$  M citrate. That the agent responsible for cessation of growth and reduction in  $Q_{KA}$  may be acetate is supported by the following experiment. Cells were grown without aeration in media containing a limiting concentration of citrate ( $2.3 \times$

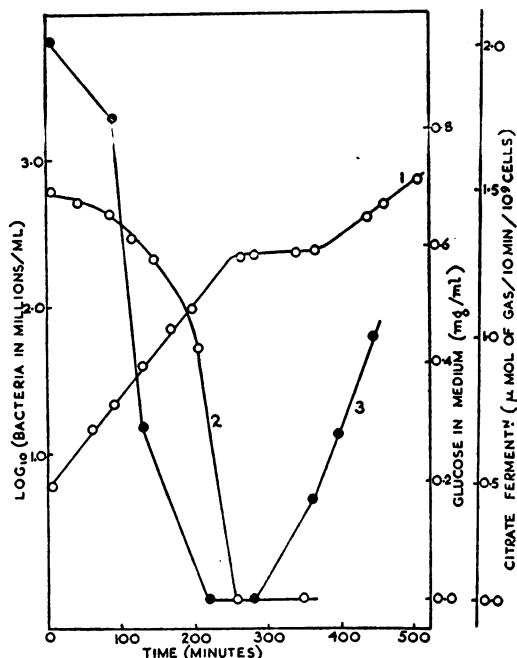


Figure 5. Diauxic growth in un-aerated citrate medium containing a limiting concentration (0.7 mg/ml) of glucose. Bacteria for inoculation were grown in un-aerated citrate medium. Growth, (1); glucose concentration in medium, (2); ability to ferment citrate, (3).

$10^{-2}$  M) to which additions of sodium acetate were made. The  $Q_{KA}$  for cells grown without acetate addition was 0.92 and for those grown in the presence of  $2.3$  and  $4.6 \times 10^{-2}$  M acetate was 0.61 and 0.16, respectively. Further experiments with cells of high initial  $Q_{KA}$  values showed that the rate of keto acid production on incubation with citrate-phosphate solution was diminished by acetate addition and that activity was not recovered merely by washing; activity, however, was regained rapidly during incubation with the complete growth medium at 37 C. Omission of

$MgSO_4$  from the medium prevented growth and recovery of activity.

From these experiments it was clear that for production of cells of high  $Q_{KA}$  it was necessary either to harvest them during the logarithmic phase or to grow them at limiting citrate concentrations.

*Effect of glucose on citrate dissimulation.* Brewer and Werkman (1939) observed that glucose, in glucose-citrate mixtures, was fermented much more rapidly by *A. indologenes* than the citrate. In figure 5 it is seen that when growth occurred without aeration in a medium containing  $0.4 \times 10^{-2}$  M glucose and  $7.1 \times 10^{-2}$  M citrate, after inoculation with cells grown anaerobically on citrate, the phenomenon of diauxie (Monod, 1942) was exhibited and the citrate in the medium was attacked only after a period of adaptation when the glucose disappeared. Measurements of the ability of the cells to ferment citrate were made for samples taken at intervals during the experiment. Gas evolution in an atmosphere of  $N_2 + 5$  per cent  $CO_2$  was measured in Warburg respirometers over a 10 minute interval; the flasks, at 37 C, contained  $10^9$  cells per ml in 3 ml citrate-phosphate solution. Citrate fermentative ability decreased rapidly during growth on glucose and was regained when cellular division recommenced. In similar experiments  $Q_{KA}$  values for cells sampled during growth decreased to zero during glucose consumption and increased again at the end of the adaptive period.

*Inhibition of citrate dissimulation by arsenite.* Complete inhibition of citrate dissimulation by 0.003 M arsenite was reported by Brewer and Werkman (1939). We found, however, that 0.0025 M  $As_2O_3$  completely inhibited pyruvate dissimulation but permitted some evolution of gas from citrate. Total gas evolution from citrate and pyruvate in the presence and absence of  $As_2O_3$  was measured for anaerobically grown cells in an atmosphere of  $N_2 + CO_2$ ; results are shown in figure 6. The total gas evolution from citrate in the presence of  $As_2O_3$  approximated to one-third of that observed in its absence (2.3 and 6.0  $\mu M$ , respectively). Determinations of pyruvate accumulation from citrate in the presence of  $As_2O_3$  gave 2.4  $\mu M$  compared with 2.3  $\mu M$  gas evolved. No pyruvate could be detected in the absence of  $As_2O_3$  at the end of the experiment. Gas evolution did not occur with  $As_2O_3$  inhibited cells when KOH was present in the center well in an atmosphere of  $N_2$ .

*Formic hydrogenlyase activity of cells grown anaerobically.* Cells growing anaerobically on citrate develop strong formic hydrogenlyase activity. This activity was estimated by measuring the hydrogen evolution in 10 min in Warburg vessels containing 1 ml sodium formate (1 per cent), 1 ml phosphate buffer, pH 7.0, and 1 ml bacterial suspension in buffer containing  $3 \times 10^9$  cells. The center well contained 0.2 ml 20

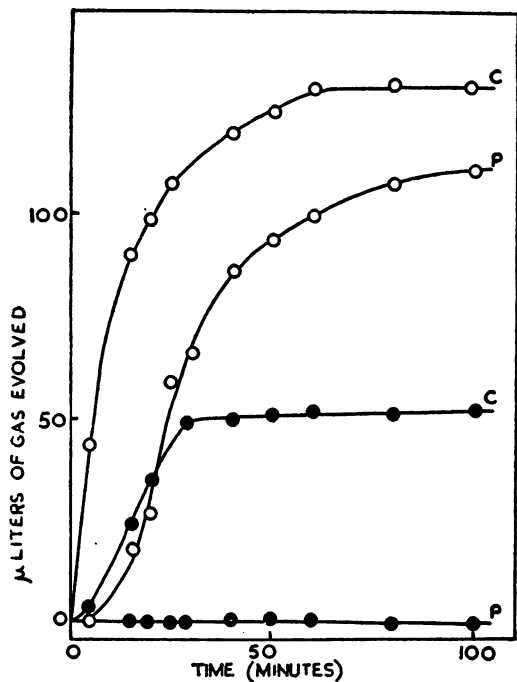


Figure 6. Total evolution of gas during fermentation of citric and pyruvic acids ( $2.5 \mu\text{M}$  of each) by cells grown anaerobically in citrate medium. Substrates: citrate, C; pyruvate, P. Addition of  $0.0025 \text{ M As}_2\text{O}_3$ , ●; no addition, ○.

per cent KOH, and the atmosphere was  $\text{N}_2$ . Cells harvested from unaerated media rapidly lost formic hydrogenlyase activity when allowed to grow with aeration. Under anaerobic conditions the activity increased during the logarithmic phase. To investigate the question of whether hydrogenlyase activity was developed simultaneously with citrate dissimilation, cells were grown with aeration, harvested, washed, and resuspended in growth medium. The culture was divided into two batches, one of which was made  $0.01 \text{ M}$  with respect to formate, and both were incubated without aeration at  $37 \text{ C}$ . Figure 7 shows that during growth in the medium con-

taining no formate hydrogenlyase activity developed after 100 min whereas in the presence of formate it developed from the start of incubation. It is apparent that the enzyme is formed in response to formate accumulating as the result of citrate breakdown.

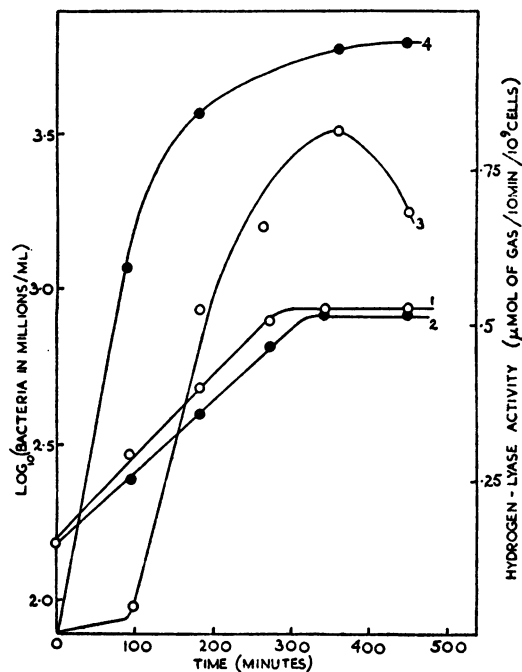


Figure 7. Development of formic hydrogenlyase activity during growth in unaerated citrate medium. Bacteria for inoculation were grown in aerated citrate medium. Growth curves: with addition of  $0.01 \text{ M}$  sodium formate to citrate medium, (2); with no addition, (1). Formic hydrogenlyase activity of cells growing in presence of  $0.01 \text{ M}$  sodium formate, (4); with no formate addition, (3).

#### DISCUSSION

The rapid production of pyruvate as sole keto acid from citrate by cells grown anaerobically accords with the mechanism proposed by Brewer and Werkman (1939) if it is assumed that oxalacetic acid is decomposed to pyruvate as rapidly as it is formed. We have isolated the 2,4 dinitrophenylhydrazone of pyruvic acid from fluids in which anaerobically grown cells had been incubated with citrate in the presence of arsenite. The method of isolation used did not differentiate between the derivatives of pyruvic and  $\alpha$ -ketoglutaric acids, but since the former was obtained pure, this supports the other evidence presented

that pyruvic is the sole keto acid produced in these circumstances. Attempts to demonstrate the accumulation of oxalacetate by cells without oxalacetic decarboxylase activity were not successful because of the difficulty experienced in obtaining such cells. Aging of suspensions held in the refrigerator at pH 4 had little effect on the active oxalacetic decarboxylase.

The evidence we have presented is also in agreement with Brewer and Werkman's suggestion that the enzyme is adaptive since it is lost during growth on glucose or on citrate in aerobic conditions. The activity is not affected by aeration in a nitrogen-free citrate medium and is lost relatively slowly during the logarithmic phase of aerobic growth. The normal route of citrate oxidation may be the tricarboxylic acid cycle since the main keto acid produced by cells harvested from aerated cultures appears to be  $\alpha$ -ketoglutarate, and is certainly not pyruvate, and keto acid accumulation is suppressed largely when air is excluded. The fact that aeration does not exclude the operation of the citrate split may accord with the observations of Baskett and Hinshelwood (1950) who concluded that two modes of aerobic growth on citrate were possible for *A. aerogenes*. We found that ability to dissimilate citrate was regained only on prolonged anaerobic incubation if the cells had been maintained in their stationary phase with aeration for a long time; such was the case for the cells used in obtaining figure 2. When harvested shortly after logarithmic growth had ceased, this lag period was much shorter and the mechanism by which citrate is oxidized consequently may be related to the lag period of the inoculating cells as Baskett and Hinshelwood (1950) found.

Our experiments on  $As_2O_3$  inhibition and formic hydrogenlyase activity together indicate that pyruvate formed by citrate dissimilation undergoes fission to formate and acetate. Accumulation of the latter inhibits the rapid formation of pyruvate, and this apparently leads to cessation of growth in anaerobic cultures with citrate unconsumed. The large increase in crop which attends aeration of these cultures (Dagley *et al.*, 1953) may result from the establishment of the alternative tricarboxylic acid cycle mechanism by which acetate would also be consumed. It may be suggested that the Pasteur effect in these cultures is dependent on the operation of alternative pathways for citrate utilization.

#### ACKNOWLEDGMENTS

We are indebted to Dr. G. A. Morrison for collaboration in the initial stages of this work and to Mrs. M. Lees and Mr. J. Smillie for their skilful technical assistance.

#### SUMMARY

The rate of keto acid accumulation when *Aerobacter aerogenes*, grown anaerobically on citrate, was aerated with citrate-phosphate solution was considerably greater than that observed when cells were grown aerobically. In the former case, pyruvic acid was the sole keto acid detected, and its production was not dependent on admission of air. For the latter cells, absence of air reduced keto acid formation while the evidence of paper chromatography showed  $\alpha$ -ketoglutaric acid to be the main product, with small amounts of pyruvic acid. Anaerobic citrate dissimilation apparently was dependent on an adaptive enzyme system whose development was suppressed by growth in aerobic conditions. Diauxic growth was exhibited by cells growing in unaerated glucose-citrate media, and during growth on glucose, enzymes responsible for citrate fermentation were suppressed. Growth in unaerated media containing concentrations greater than  $5.4 \times 10^{-2}$  M citrate ceased with citrate unconsumed, and the ability of the cells to produce pyruvate rapidly from citrate was impaired. These phenomena are attributed to the action of accumulated acetic acid. In the presence of 0.0025 M  $As_2O_3$ , pyruvate accumulated during citrate dissimilation and an approximately equimolar amount of  $CO_2$  was evolved. The formic hydrogenlyase activity of cells grown on citrate has been studied.

#### REFERENCES

- AJL, S. J., AND WERKMAN, C. H. 1949 On the mechanism of carbon dioxide replacement in heterotrophic metabolism. *J. Bact.*, **57**, 579-593.
- BASKETT, A. C., AND HINSHELWOOD, SIR C. 1950 The utilization of carbon sources by *Bact. lactis aerogenes*. I. General survey. *Proc. Roy. Soc. (London)*, **B**, **136**, 520-535.
- BREWER, C. R., AND WERKMAN, C. H. 1939 The anaerobic dissimilation of citric acid by *Aerobacter indologenes*. *Enzymologia*, **6**, 273-281.
- DAGLEY, S., DAWES, E. A., AND FOSTER, SHELAGH M. 1953 Influence of pH value and aeration

- on the growth of *Aerobacter aerogenes* and *Bacterium coli* in defined media. *J. Gen. Microbiol.*, **8**, 314-322.
- DAGLEY, S., DAWES, E. A., AND MORRISON, G. A. 1950 Factors influencing the early phases of growth of *Aerobacter aerogenes*. *J. Gen. Microbiol.*, **4**, 437-447.
- DAGLEY, S., DAWES, E. A., AND 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**, 433-441.
- DAGLEY, S., FEWSTER, MONA E., AND HAPFOLD, F. C. 1952 The bacterial oxidation of phenylacetic acid. *J. Bact.*, **63**, 327-336.
- DEFFNER, M., AND FRANKE, W. 1939 Der Abbau der Citronensäure durch Bakterien. *Ann. Chem., Justus Liebig*, **541**, 85-117.
- FOWLER, C. B. 1951 The relationship between fermentation and enzymatic adaptation. *Biochim. et Biophys. Acta*, **7**, 563-573.
- FRIEDEMANN, T. E., AND HAUGEN, G. E. 1943 Pyruvic acid. II. The determination of keto acids in blood and urine. *J. Biol. Chem.*, **147**, 415-441.
- KORKES, S., STERN, J. R., GUNSALUS, I. C., AND OCHOA, S. 1950 Enzymatic synthesis of citrate from pyruvate and oxalacetate. *Nature*, **166**, 439-440.
- KREBS, H. A. 1950 The tricarboxylic acid cycle. Harvey Lectures, Series XLIV, 165-199.
- MONOD, J. 1942 Recherches sur la croissance des cultures bactériennes. Hermann & Cie., Paris.
- NELSON, N. 1944 A photometric adaptation of the Somogyi method for the determination of glucose. *J. Biol. Chem.*, **153**, 375-380.
- STONE, R. W., AND WILSON, P. W. 1952 Respiratory activity of cell-free extracts from *Azotobacter*. *J. Bact.*, **63**, 605-617.