Pyruvate Accumulation in Growth-Inhibited Cultures of *Aerobacter aerogenes*

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1. Accumulation of pyruvate occurs during the early stages of exponential growth of aerobic, anaerobic and static cultures of a strain of *Aerobacter aerogenes*. In normal cultures of this organism the content of pyruvate increases until most of the glucose of the medium has been consumed, and then declines rapidly. The presence of unconsumed sugar is not the sole reason for the accumulation of keto acid, since this is unaffected by the addition of extra glucose to either exponential-phase or stationary-phase cultures. 2. In aminopterin-inhibited cultures, the rate of glucose utilization is decreased greatly, and pyruvate continues to accumulate throughout the period of incubation. This prolonged phase of accumulation appears to be a consequence of the growth inhibition, and not to a specific action of aminopterin on the phosphoroclastic breakdown of pyruvate, since it occurs also when growth is restricted by the antibiotics streptomycin, chloramphenicol and neomycin. 3. A possible explanation is suggested for the accumulation of pyruvate in the inhibited cultures.

In the preceding paper (Webb, 1968) it was reported that cells from static cultures of *Aerobacter aerogenes*, in which growth was inhibited by aminopterin, were deficient in some cofactor necessary for the anaerobic phosphoroclastic breakdown of pyruvate to acetate, carbon dioxide and hydrogen, and for formate-pyruvate-exchange activity. Oxidative and other pathways of pyruvate metabolism, however, were unaffected. Although the phosphoroclastic route probably contributes significantly to the metabolism of pyruvate in static cultures of this organism, particularly during the exponential phase when growth is essentially anaerobic, the inhibition of this reaction seemed unlikely to be the sole reason for the excessive accumulation of the keto acid in the aminopterin-treated cultures. Further studies have therefore been made of the production of pyruvate during normal growth of *A. aerogenes* in a simple medium with glucose as the sole source of organic carbon, and in cultures in which growth has been inhibited by aminopterin and by certain antibiotics. The results of this work are summarized in this paper.

MATERIALS AND METHODS

Glucose was determined by the method of Nelson (1944). Other analytical procedures and the conditions for the cultures of *A. aerogenes* were as described in the preceding paper (Webb, 1968), the chemically defined medium being used in all of the present experiments. Aminopterin,

chloramphenicol (Parke, Davis and Co., Detroit, Mich., U.S.A.), neomycin (Upjohn Chemical Co., Kalamazoo, Mich., U.S.A.) and streptomycin (Upjohn Chemical Co.) were gifts from Dr W. J. Nickerson, Institute of Microbiology, Rutgers University, New Brunswick, N.J., U.S.A.

RESULTS

Some accumulation of pyruvate occurred during exponential growth in static (Fig. 1), aerated (Fig. 2) and anaerobic (Table 1) control cultures of *A. aerogenes*. In each type of culture, the pyruvate concentration reached a maximum (0.7–1.0 $\mu\text{mole/ml.}$) immediately before the end of the exponential phase, when about 70% of the glucose had been consumed, and then declined rapidly. Anaerobically, the decrease in pyruvate content was not accompanied by a parallel increase in that of lactate. Pyruvate alone was unable to support growth of static and aerobic cultures of *A. aerogenes*, and in media in which the glucose content was decreased progressively the addition of pyruvate in amounts sufficient to maintain the total carbon content at a constant level failed to influence either the rate of growth or the final cell density.

When growth of static cultures was inhibited by the addition of aminopterin (0.114 $\mu\text{mole/ml.}$) in the early exponential phase, the pyruvate concentration increased throughout the period of incubation and at 24 hr. was several times greater than the maxima in the controls (Fig. 1). In similarly treated

Fig. 1. Growth (□), glucose utilization (○), pyruvate accumulation (●) and acetylmethylcarbinol synthesis (▲) in (a) aminopterin-treated and (b) control static cultures of *A. aerogenes* at 37°. The experimental conditions and analytical methods were as described in the Materials and Methods section of this and of the preceding paper (Webb, 1968). Aminopterin (0.114 μmole/ml.) was added to (a) at 4.25 hr.

Fig. 2. Growth (Δ), glucose utilization (○) and pyruvate content (●) in an aerated culture of *A. aerogenes* at 37°. The experimental methods were as described for Fig. 1.

Table 1. *Glucose utilization and pyruvate and lactate accumulation in normal and aminopterin-treated anaerobic cultures of A. aerogenes*

Cultures were grown at 37° in the chemically defined medium, which was agitated continuously with a stream of N₂ + CO₂ (95:5). Aminopterin (0.114 μmole/ml.) was added to one culture after 5½ hr. Analyses were made as described in the Materials and Methods section of this and of the preceding paper (Webb, 1968).

Age of culture (hr.)	Control				Aminopterin-treated culture			
	Cell density (μg. dry wt. of organisms/ml.)	Residual glucose (μmoles/ml.)	Pyruvate (μmole/ml.)	Lactate (μmoles/ml.)	Cell density (μg. dry wt. of organisms/ml.)	Residual glucose (μmoles/ml.)	Pyruvate (μmoles/ml.)	Lactate (μmoles/ml.)
2.0	11	—	—	—	11	—	—	—
3.25	19	—	—	—	19	—	—	—
4.7	35	—	—	—	35	—	—	—
5.5	52	—	0.21	—	49	—	0.22	—
6.5	85	18.0	0.44	1.3	61	21.2	0.24	0.5
7.25	112	15.8	0.60	1.3	64	—	0.27	1.1
8.0	137	13.7	0.76	2.2	72	19.8	0.35	1.4
8.75	241	8.7	0.91	2.9	76	18.5	0.43	1.6
9.5	342	4.0	0.96	3.9	86	16.4	0.56	1.9
10.25	343	—	0.42	4.0	82	13.8	0.68	2.3
11.0	345	1.6	0.18	3.8	82	13.3	0.79	3.0
11.75	—	1.1	0.12	3.7	—	11.2	0.90	3.1
13.5	326	1.0	0.08	3.8	90	10.1	1.12	—
26.0	—	0.6	0.04	3.6	—	8.5	1.64	4.8

Fig. 3. Growth (○), glucose utilization (▲) and pyruvate content (●) in control (----) and aminopterin-treated (—) static cultures of *A. aerogenes* in a chemically defined medium of restricted N content (24 μg. of ammonia N/ml.) at 37°. The addition of aminopterin (0.11 mM) was made at 5 hr. Details of the medium are given in the text and the analytical methods were as described for Fig. 1.

anaerobic cultures the contents of both pyruvate and lactate increased slowly, but continuously, after the addition of aminopterin. In the inhibited static cultures the rates of acetylmethylcarbinol accumu-

lation were less than in the control cultures (Fig. 1), although the final contents, when expressed relative to the cell concentrations (dry wt. of organisms/ml.) were greater. No significant difference was found

between the rates of pyruvate oxidation by cells that were harvested from the control and aminopterin-treated cultures at different times throughout the 24 hr. incubation period. In cells from both cultures the oxidation rate ($\mu\text{l.}$ of oxygen consumed/hr./mg. dry wt. of organisms) decreased by 20–25% during the first 6–9 hr. of incubation. Subsequently, activity increased to become approximately the same as that of cells from the initial lag phase. This readjustment occurred more rapidly in cells from the control than from the aminopterin-treated cultures.

The exogenous concentration of pyruvate increased after the addition of extra glucose (2.2 and 22 $\mu\text{moles/ml.}$) to fully grown static cultures. The sugar was utilized rapidly and the pyruvate content reached a maximum (0.27–0.5 $\mu\text{mole/ml.}$) after about 2 hr. at 37°. Neither the rates of glucose consumption nor the changes in pyruvate content were altered by high concentrations of aminopterin (0.17 and 0.62 $\mu\text{mole/ml.}$).

The accumulation of pyruvate, although apparently related to the presence of unconsumed glucose, was not determined solely by the sugar content of

the medium. Thus, when extra glucose (125 $\mu\text{moles/ml.}$) was added to one of a pair of identical static cultures of *A. aerogenes*, the initial rates of growth in the two cultures were the same. With the glucose supplement, however, growth continued, though at a slightly decreased rate, after the normal culture had entered the stationary phase, and ultimately reached a cell density about 1.8-fold greater. In both cultures, pyruvate accumulated to reach approximately the same maxima (1.0 and 1.4 $\mu\text{moles/ml.}$) at the same time, although the content of residual glucose in the supplemented culture was still 4½-fold greater than the initial concentration in the normal medium.

Half-maximum growth was obtained by decreasing the concentration of ammonia nitrogen in the medium from 584 $\mu\text{g.}$ to 24 $\mu\text{g.}$ of nitrogen/ml. Although pyruvate also accumulated during growth under these conditions, the maximum concentration was attained much later (i.e. in the stationary phase) and was lower than in cultures with the higher initial nitrogen content (Fig. 3, cf. Figs 1 and 2). Aminopterin, added in the early exponential phase of these cultures, inhibited growth, decreased

Fig. 4. Effect of certain antibiotics on growth (▲—▲), glucose utilization (○—○) and pyruvate production (●—●) by *A. aerogenes* at 37°. Additions were made as follows: (a) none; (b) streptomycin sulphate (200 $\mu\text{g./ml.}$) at 3–25 hr.; (c) streptomycin sulphate (50 $\mu\text{g./ml.}$) at 1.75 hr.; (d) chloramphenicol (100 $\mu\text{g./ml.}$) at 1.75 hr.; (e) neomycin (100 $\mu\text{g./ml.}$) at 2 hr.; (f) streptomycin sulphate (50 $\mu\text{g./ml.}$) and aminopterin (90 $\mu\text{g./ml.}$) at 1.5 hr. The analytical methods were as described for Fig. 1.

the rate of glucose utilization and delayed the onset of pyruvate accumulation. Ultimately, however, the pyruvate content exceeded the maximum amount of the controls.

As the maximum concentration of pyruvate was reached in the stationary phase of cultures with the restricted nitrogen content, it was possible to determine the effect of aminopterin on cultures that were actively accumulating the keto acid, but in which growth had ceased. Such a culture was divided into two fractions, one of which was treated with aminopterin (95 μ moles). On further incubation, the rates of glucose utilization and of pyruvate accumulation to the same maximum were identical in both cultures.

The effects of aminopterin on normal static cultures of *A. aerogenes* were duplicated by streptomycin, chloramphenicol and neomycin (Figs. 4b, 4c, 4d and 4e). Thus, in all cultures to which the antibiotics were added early in the exponential phase, pyruvate continued to accumulate throughout the subsequent incubation period. In the presence of neomycin (Fig. 4c) and the higher concentration of streptomycin (Fig. 4b) glucose utilization was inhibited completely. The continued increase in pyruvate content under these conditions therefore must have been due to the metabolism of endogenous substrates or the products of cell lysis (the occurrence of which was shown by the progressive decrease in cell density after 4.6 hr.) or both. At the lower concentration of streptomycin (Fig. 4c) glucose metabolism continued, whereas the rate of pyruvate accumulation and the final content (7 μ moles/ml. at 24 hr.) were both increased. The combination of aminopterin (90 μ g./ml.) with streptomycin (50 μ g./ml.) was more inhibitory to growth than the antibiotic alone, but had little effect on the rate of pyruvate formation (Fig. 4f).

DISCUSSION

Accumulation of small amounts of pyruvate during the exponential phase of growth has been observed previously in aerated cultures of *A. aerogenes* (Dagley, Dawes & Morrison, 1951; Dagley, Dawes & Foster, 1953) and of *Bacillus cereus* (Nakata & Halvorson, 1960). With the strain of *A. aerogenes* used in the present work, such accumulation appears to be a normal event in aerobic, anaerobic and static cultures in a simple chemically defined medium, and continues until most of the glucose has been utilized. The presence of un-consumed sugar, however, is not the sole reason for the accumulation of the keto acid, since the addition of a large excess of glucose, either during exponential growth or to stationary-phase cultures, neither extends the period of accumulation nor increases the pyruvate content above the normal

maximum. From this evidence, the continued production of pyruvate throughout the period of incubation of cultures in which growth is inhibited by aminopterin cannot be due simply to the decreased rate of glucose utilization, and hence to the maintenance of a sugar concentration that favours the formation of the keto acid.

In addition to any direct effect of aminopterin on pyruvate metabolism, as for example on phosphoroclastic breakdown (Webb, 1968), accumulation of the keto acid appears to be generally associated with the inhibition of growth of *A. aerogenes* in a salts-glucose medium, since it occurs also in the presence of bacteriostatic concentrations of chemically unrelated antibiotics. Although the primary action of both chloramphenicol and streptomycin is on protein synthesis (Valquez, 1966; Brock, 1966), there are many reports, particularly with regard to the latter antibiotic (see review by Oginsky, 1953), of the inhibition of oxidative metabolism and various catabolic reactions by these compounds. Particularly relevant to the present work are the observations of Padron (1960) on the inhibition of pyruvate metabolism by chloramphenicol in *Staphylococcus aureus*, of Barkulis (1953) on the inhibition of pyruvate fermentation and of the phosphoroclastic reaction, but not of glucose utilization, by dihydrostreptomycin in *Escherichia coli*, and of Jones & Morrison (1963) on the inhibition of the production of formate from pyruvate by tetracycline in unaerated cultures of *A. aerogenes*. Jones & Morrison (1963) regard this effect of tetracycline, the bacteriostatic action of which is due probably to the specific inhibition of protein synthesis (Franklin, 1966), as indirect and to result from interference with a hydrogen-transfer reaction that is by-passed in aerated cultures.

Accumulation of excess of pyruvate occurs when aminopterin is added to actively growing cultures, but not to stationary-phase cultures or to suspensions of resting cells. As Brock (1966) has emphasized with regard to antibiotic experiments, there is little value in the comparison of such treated cultures with untreated controls at a specific time, since in the latter there is a progressive increase in all cell constituents. Wright & Lockhart (1965) have shown that the composition of *E. coli* varies both with the growth rate and the identity of the limiting nutrient, and confirmed that cellular composition reflects the physiological adjustment of a microbial population to its environment. Alterations in composition occur in *A. aerogenes* as a result of the inhibition of growth by aminopterin (Webb, 1958) and, as discussed by Jackson (1958) with regard to the effects of streptomycin on bacterial respiration, are probably accompanied by changes in the balance of cellular enzymes. *A. aerogenes*, however, has a multiplicity of mechanisms for

pyruvate metabolism, only one of which appears to be inhibited by aminopterin (Webb, 1968). The present results, for example, show the continued production of acetylmethylcarbinol in the aminopterin-treated cultures and no significant alteration in the activity of the pyruvate oxidation system. Thus a change in enzyme balance seems unlikely to be responsible for pyruvate accumulation.

Possibly the excess of pyruvate in cultures in which growth is restricted by aminopterin, or by inhibitors of protein synthesis such as chloramphenicol and streptomycin, is related to the decreased requirements for C₄ compounds. Since unadapted glucose-grown *A. aerogenes* cells do not utilize acetate (Ravin, 1952; Hadjipetrou, Gerrits, Teulings & Stouthamer, 1964), whereas carbon dioxide is an essential metabolite (Ajl & Werkman, 1948), the main route of the synthesis of C₄ compounds is probably through carbon dioxide fixation. If this reaction is particularly sensitive to end-product repression, inhibition of growth with the consequent decrease in the rate of utilization of C₄ intermediates, might lead to an accumulation of pyruvate, either directly or through the decreased requirements for phosphoenolpyruvate. On this hypothesis pyruvate would not be expected to accumulate when aminopterin is added to either

stationary cultures or resting suspensions, since under these conditions the requirements for C₄ compounds would be the same for both treated and untreated cells.

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