Pyruvate Metabolism by Aminopterin-Inhibited *Aerobacter aerogenes*

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1. The synthesis and utilization of both alanine (by reductive amination, oxidative deamination and transamination) and valine (by transamination only) in *Aerobacter aerogenes* are unaffected by aminopterin. These amino acids, which accumulate in aminopterin-treated cultures of this organism, are therefore considered to be formed as secondary products from the excess of pyruvate that also accumulates. 2. Oxidative metabolism of pyruvate and the synthesis of acetylmethylcarbinol by *A. aerogenes* cells are unaltered by growth in the presence of aminopterin. 3. Cells from static and anaerobic cultures that have been treated with the folic acid antagonist in the early exponential phase have a decreased ability to cleave pyruvate to acetate and formate, and to effect the exchange of formate with the carboxyl group of pyruvate. 4. 3-Methyl-2-oxobutanoate, the keto acid precursor of valine, cannot replace pyruvate as substrate in either the phosphoroclastic or the exchange reaction.

Inhibition of growth in static cultures of *Aerobacter aerogenes* by the folic acid antagonist, aminopterin, leads to the accumulation of alanine and valine in addition to other metabolites (Webb, 1958). On recovery from the inhibition, these amino acids are utilized rapidly, and both contribute to the synthesis of leucine.

Further studies have indicated that the above amino acids are formed as secondary products from pyruvate and 3-methyl-2-oxobutanoate, the former of these keto acids, from which the latter is known to be derived, being the primary intermediate to accumulate. These observations led to an investigation of the effects of aminopterin on the different pathways of pyruvate metabolism by *A. aerogenes*, the results of which are summarized in this paper. In addition, certain parallel studies are reported on the metabolism of 3-methyl-2-oxobutanoate. It is shown that cells from aminopterin-treated cultures are deficient in some factor that is necessary for phosphoroclastic and formate-pyruvate-exchange activity, and have a decreased ability to cleave pyruvate to acetate and formate. 3-Methyl-2-oxobutanoate cannot replace pyruvate in either the phosphoroclastic or the exchange system, and no evidence has been obtained for the direct production of formate from this substrate.

MATERIALS AND METHODS

Growth of cultures. The source of the *A. aerogenes* strain and the conditions for its maintenance have been given previously (Webb, 1958). Static cultures were grown at 37° either in Monod's (1942) chemically defined medium or in a

solution of 2% (w/v) peptone (British Drug Houses Ltd., Poole, Dorset), 0.5% (w/v) NaCl and 0.2% (w/v) glucose. The former medium was used in all experiments in which aminopterin was added to growing cultures. Such additions were made in the early exponential phase (Webb, 1958). Aerobic cultures were grown at 37° either on a mechanical shaker or with enforced aeration. Anaerobic cultures were grown in an atmosphere of N₂ + CO₂ (95:5), the gas mixture being used to agitate the medium.

Cells from all cultures were harvested after 16–20 hr., washed three times in 0.15M-NaCl and, for the metabolic studies, then resuspended (10mg. dry wt./ml.) in 0.15M-NaCl. Cells grown in the presence of aminopterin are termed 'AM cells'. Dry weights were determined turbidimetrically after appropriate dilution, standard curves being prepared from cells grown under the corresponding conditions.

Preparation of cell homogenates and extracts. Washed cells were disintegrated either in the Hughes (1951) press or by grinding with alumina (McIlwain, 1948). In the latter procedure the viscous suspension of *A. aerogenes* organisms was first centrifuged for 60–90 min. at 44330g (Spinco model L centrifuge, no. 21 rotor) to obtain the cells as a compact pellet. After disintegration, the homogenate was centrifuged at 7700g at 4° and the supernatant stored at 0°. Further centrifugation of this fraction for 1 hr. at 105400g at 4° (Spinco model L centrifuge, no. 40 rotor) gave a clear viscous extract. Because of the high extracellular polysaccharide content of *A. aerogenes* it was more convenient in some large-scale preparations to freeze-dry a dilute cell suspension (10mg. dry wt. of organisms/ml.) and to moisten the powder (1g.) with water (3ml.) before addition of the alumina (10g.).

Manometry. Measurements of utilization of O₂ with air as the gas phase, and of H₂ and CO₂ production in N₂, were made in the conventional Warburg apparatus at 37°. Specific details of the experimental systems, other than

those summarized below, are given in the legends of the Tables and Figure. Phosphate buffers were used in all systems.

Assay of formate dehydrogenase. The activities of cell suspensions, homogenates and extracts were determined manometrically at 37° in the presence of methylene blue (20 μ moles) as hydrogen acceptor and N₂ as the gas phase (Wolin & Lichstein, 1956).

Synthesis of acetylmethylcarbinol. The experimental system was incubated at 37° and contained, in a volume of 6.5 ml., potassium phosphate buffer, pH 6.1 (200 μ moles), sodium pyruvate (300 μ moles) and *A. aerogenes* cells (20 mg. dry wt. of organisms). At intervals portions (1.0 ml.) were diluted with 0.1N-H₂SO₄ (5.0 ml.) and centrifuged. Suitable volumes of the supernatant fraction were analysed for acetylmethylcarbinol. When homogenates were used in place of intact cells, the acetylmethylcarbinol-synthesizing system was separated from pyruvate oxidase and the particulate formate dehydrogenase (Wolin & Lichstein, 1956) by centrifugation. The system mediating the formate-pyruvate-exchange reaction, which remained in the supernatant fraction, was unstable and was inactivated within 24 hr. at 4°. The cell-free extracts then catalysed an almost quantitative conversion of pyruvate into acetylmethylcarbinol.

Formate-pyruvate exchange. The assay system was similar to that of Chantrenne & Lipmann (1950) and contained the cell-free extract (0.1–0.2 ml.), sodium pyruvate (40 μ moles), CoA (10 μ g.), cysteine (10 μ moles), phosphate buffer, pH 7.4 (100 μ moles) and sodium [¹⁴C]formate (50 μ moles; 4000 counts/min./ μ mole) in a total volume of 1.5 or 2.0 ml. Aminopterin, when added, was dissolved in the buffer. The gas phase used was N₂, although control experiments indicated that the reaction was not affected appreciably by air. After incubation for 45 min. at 37°, protein was removed with 5% (w/v) trichloroacetic acid and the pyruvic acid in the supernatant fractions precipitated as the 2,4-dinitrophenylhydrazone. The precipitates were washed as described by Chantrenne & Lipmann (1950) and dissolved in ethanol. Portions of these solutions were diluted to a suitable volume with 2% (w/v) Na₂CO₃ solution and analysed by the method of Friedemann & Haugen (1943). The remainder of each ethanolic solution was adjusted to contain 10 μ moles of the 2,4-dinitrophenylhydrazone/ml. and samples (1.0 ml.) were then plated on aluminium planchets (3.5 cm. diam.). Radioactivity was measured with a scintillation counter type 663A and a scaler type 1700 (Isotope Developments Ltd.), sufficient counts being collected to ensure a counting error of less than 3%. With the uniform plating procedure a correction was applied for background, but not for self-absorption.

Analytical methods. Pyruvic acid and other keto acids were determined by the method of Friedemann & Haugen (1943). The 2,4-dinitrophenylhydrazones of pyruvate and of 3-methyl-2-oxobutanoate were separated from that of 2-oxoglutarate by extraction into toluene. Experimentally determined factors were used to correct for interference by aminopterin in the colorimetric procedure, and for the incomplete extraction of the 2,4-dinitrophenylhydrazone of 3-methyl-2-oxobutanoate from organic solvents by 10% (w/v) Na₂CO₃. Individual components of mixtures of keto acids were separated also by chromatography of the 2,4-dinitrophenylhydrazones on buffered paper (Kun & Garcia-Hernandez, 1957).

Lactic acid was measured by the method of Barker & Summerson (1941).

Acetylmethylcarbinol and diacetyl were determined as described by Westerfeld (1945).

Nitrogen was estimated by the micro-Kjeldahl procedure and protein by the biuret method (Layne, 1957), with bovine serum albumin as standard.

Chemicals. Melting points and boiling points are uncorrected.

3-Methyl-2-oxobutanoic acid (m.p. 31°, b.p. 65°/10 mm. Hg) was obtained in 50% yield from 4-isopropylidene-2-phenyloxazoline by hydrolysis in 12N-HCl (Ramage & Simonsen, 1935). The acid was converted into the sodium salt as described by Meister (1951).

Tetrahydrofolic acid was prepared by hydrogenation of folic acid (General Biochemicals Inc., Chagrin Falls, Ohio, U.S.A.) at room temperature in the apparatus described by Noller & Barusch (1942) in the presence of Adams catalyst (Linstead, Elvidge & Whalley, 1955). To avoid the formation of colloidal platinum, the solution of folic acid (10 mm) in aq. NaHCO₃ (20 mm) was added from a side arm of the hydrogenation vessel after the preliminary reduction of the aqueous suspension of the catalyst was complete (cf. O'Dell, Vandenbelt, Bloom & Pfiffner, 1947).

Aminopterin was a gift from Dr W. J. Nickerson (Institute of Microbiology, Rutgers University, New Brunswick, N.J., U.S.A.).

RESULTS

In comparison with the controls, filtrates from cultures of AM cells contained high concentrations of pyruvate (1.5–2.3 mm) and 3-methyl-2-oxobutanoate (0.75–1.2 mm), together with other metabolites derived from pyruvate (e.g. lactate, acetaldehyde, acetylmethylcarbinol, diacetyl, butane-2,3-diol and acetolactate). Both pyruvate and 3-methyl-2-oxobutanoate were converted readily into the corresponding amino acids by reversible transamination reactions in the presence of crude cell-free extracts, alanine being formed from pyruvate and L-glutamate, L-valine and L-leucine, and valine from 3-methyl-2-oxobutanoate and L-glutamate, L-leucine and L-alanine. Extracts of both control and AM cells were equally effective in the catalysis of these reactions, which have been described previously in *A. aerogenes* and in other bacteria (Adelberg & Umbarger, 1953; Meister, 1955), and the addition of aminopterin (4.5 μ moles) to any system was not inhibitory.

In suspensions of *A. aerogenes* cells, reductive amination also contributed to the synthesis of alanine from pyruvate, but not to that of valine from 3-methyl-2-oxobutanoate. Neither reductive amination of pyruvate, nor oxidative deamination of L-alanine, was affected by aminopterin.

Effect of aminopterin on pyruvate metabolism. The above observations indicated that alanine and valine, which are accumulated by *A. aerogenes* in the presence of aminopterin (Webb, 1958), were formed as secondary products from the excess of

keto acids, and that pyruvate was the first intermediate to accumulate in the treated cultures. The effect of aminopterin on various pathways of pyruvate metabolism by *A. aerogenes* was therefore investigated.

With either suspensions or homogenates of washed cells of equal dry weight from control and aminopterin-treated cultures, no significant or consistent differences were observed in the rates of the following reactions of pyruvate: (1) oxidation to acetate; (2) dismutation to lactate, acetate and carbon dioxide (Juni, 1952); (3) conversion into acetylmethylcarbinol and butane-2,3-diol.

Also, these reactions were unaffected by the addition of aminopterin (4.5 μ moles) to the experimental system. Phosphoroclastic activity, however, was depressed significantly in AM cells.

At pH 7.4, phosphoroclastic breakdown to acetate and formate (Lipmann, 1946) was the main pathway of anaerobic pyruvate metabolism by *A. aerogenes*, the production of both lactate and acetylmethylcarbinol being suppressed above pH 7.0. As the formate that was liberated in this reaction was decomposed quantitatively by formate hydrogenlyase, the cleavage reaction could be followed manometrically by the evolution of hydrogen. With whole cells and homogenates the rate of hydrogen production from pyruvate (10 μ moles) was initially zero, but increased rapidly during the first 10–15 min. of incubation and became identical with that with formate (10 μ moles) as substrate, the metabolism of which did not show an initial lag (cf. Lascelles, 1948). The intermediary formation of acetyl phosphate during pyruvate cleavage by suspensions of cells from control cultures was not detected by the method of Lipmann & Tuttle (1945), even in the presence of sodium fluoride (50 μ moles). As lithium acetyl phosphate was hydrolysed rapidly by suspensions and extracts of *A. aerogenes*, it seems that in this organism, as in *Escherichia coli* (Lipmann, 1946), the high activity of acetyl phosphatase prevented the accumulation of acetyl phosphate.

The phosphoroclastic system was equally active in static and fully anaerobic cultures of *A. aerogenes*, but was deficient in cells that were grown with aeration (Table 1). When these aerobically grown cells were used to inoculate static cultures the capacity for anaerobic pyruvate metabolism was acquired rapidly, and was maximal at the end of exponential growth (Table 1). The activity of formate hydrogenlyase increased in parallel with that of the phosphoroclastic system (Table 1).

The addition of aminopterin (0.13 mM) in the early exponential phase (cell density 55–60 μ g. dry wt. of organisms/ml.) of anaerobic cultures of *A. aerogenes* inhibited growth by at least 66%. Phosphoroclastic activity, as measured by the evolution of hydrogen

Table 1. *Metabolism of pyruvate by the phosphoroclastic system, and of formate by formate hydrogenlyase, during static and anaerobic growth of A. aerogenes*

The primary cultures for Expts. 1 and 2 were grown at 37° with vigorous aeration for 9½ hr. and in stationary flasks for 16 hr. respectively. Portions (275 ml.) of these cultures were transferred to fresh medium at 37°. In Expt. 1 the new culture was maintained without agitation, and in Expt. 2 it was gassed continuously with N₂+CO₂ (95:5). At intervals samples were removed, and these, together with the remainder of each primary culture, were centrifuged. After being washed in 0.15 M-NaCl, the phosphoroclastic and formate hydrogenlyase activities of the cells were measured manometrically by the evolution of H₂ from pyruvate and formate at 37°. Each Warburg flask contained, in a total volume of 2.0 ml., 100 μ moles of phosphate buffer, pH 7.0, cell suspension (5 mg. dry wt. of organisms) and substrate (10 μ moles), the last-named being added from the side arm after equilibration. CO₂ was absorbed by 5 N-NaOH (0.2 ml.) in the centre well, and 100% N₂ was used as the gas phase.

Substrate ... Age of culture (hr.)	Maximum rate of H ₂ evolution (μ l./hr./mg. dry wt. of organisms)		
	Expt. 1		Expt. 2
	Formate	Pyruvate	Pyruvate
0 (inoculum)	1.5	3.3	71.8
3	37.2	41.1	56.8
5	64.6	68.0	65.8
7	77.0	87.4	80.0
9	66.8	71.5	—
11	—	—	86.5
13	54.8	64.3	—

from pyruvate in cells from the stationary phase of these cultures, varied between 0% and 44% of that of the corresponding controls and was inhibited further (33–50%) by aminopterin (4.5 μ moles; Fig. 1). Phosphoroclastic activity decreased after the addition of aminopterin to exponential-phase cultures, and this eliminated the possibility that the effects observed with cells from the stationary phase were due to an inhibition of adaptation to the anaerobic pathway (Table 2). It was also established that aminopterin affected primarily the cleavage of pyruvate, and not the formate hydrogenlyase system (Table 2). Metabolism of formate by formate dehydrogenase also was not inhibited in AM cells. Inhibition of the phosphoroclastic system in AM cells resulted in the metabolism of a greater proportion of pyruvate to acetylmethylcarbinol.

The evolution of hydrogen from pyruvate by homogenates of normal *A. aerogenes* cells from static

Fig. 1. Phosphoroclastic breakdown of pyruvate by cells from normal and aminopterin-treated anaerobic cultures of *A. aerogenes*. Evolution of H_2 from the liberated formate was measured. Each Warburg flask contained potassium phosphate buffer, pH 7.0 (200 μ moles), the cell suspension (5 mg. dry wt. of organisms) and 5N-NaOH (0.2 ml.) in the centre well. Sodium pyruvate (12 μ moles) was added from the side arm after equilibration. The total fluid volume was 2.2 ml., the temperature 37° and the gas phase N_2 . Aminopterin (4.5 μ moles), when present, was dissolved in the buffer. The cells were harvested from control and aminopterin-treated (0.12 μ mole/ml.) anaerobic cultures after 15 hr. at 37°. ●, Control cells; ▲, cells from aminopterin-treated culture; ○, control cells + aminopterin; △, cells from treated culture + additional aminopterin.

cultures was inhibited by 1.7 mM-aminopterin (Table 3), a concentration usually without effect on the activity of the intact organisms. In both the experimental and control systems little pyruvate remained unconsumed when hydrogen evolution ceased, and the synthesis of acetylmethylcarbinol was low (Table 3). Homogenates of AM cells exhibited little or no phosphoroclastic activity when assayed either alone or in the presence of folic acid (9 μ moles), tetrahydrofolic acid (7 μ moles), thiamine (1 μ mole), thiamine pyrophosphate (1 μ mole), CoA (0.1 μ mole), cysteine (0.2 μ mole), NAD (1 μ mole), FAD (1 μ mole), ATP (1 μ mole), pantothenic acid (1 μ mole), biotin (4 mg.) and pyridoxal phosphate (1 μ mole). Activity, however, was restored by a saline extract that was obtained from normal cells at 100° (Table 4). Such extracts also reversed the inhibition of phosphoroclastic activity in whole AM cells, and prevented the action of aminopterin in homogenates of control cells.

When formate hydrogenlyase was removed from homogenates of *A. aerogenes* cells by centrifugation at 105 400g, exchange of formate with the carboxyl group of pyruvate could be demonstrated in the residual supernatant fraction. This activity, as measured by the exchange of [^{14}C]formate with the carboxyl group of pyruvate, was decreased significantly either by growth of *A. aerogenes* in the presence of aminopterin (Table 5) or by the addition of the folic acid antagonist to extracts of normal cells (Table 6).

Metabolism of 3-methyl-2-oxobutanoate. All preparations of this keto acid were oxidized extremely slowly by cells from static cultures of *A. aerogenes* grown in either the peptone-water or chemically defined medium. The maximum rate of oxygen

Table 2. Inhibition by aminopterin of the phosphoroclastic breakdown of pyruvate during anaerobic growth of *A. aerogenes*

Cells from a fully grown static culture in Monod's (1942) medium (200 ml.) were used to inoculate 3l. of the same medium, which was incubated anaerobically at 37°. In the early exponential phase (cell density 60 μ g. dry wt. of organisms/ml.) one-third of the culture was centrifuged and the washed cells were stored at 4°. The remainder of the culture was divided into two fractions, both of which were incubated anaerobically and to one of which aminopterin (0.126 μ mole/ml.) was added. Cells from both of these cultures were harvested after a further 15 hr. The phosphoroclastic and formate hydrogenlyase activities of these cells, together with those of cells of the inoculum and the early exponential phase of growth, were then determined manometrically as described in Table 1.

Source of cells	Rate of H_2 evolution (μ l./hr./mg. dry wt. of organisms)			
	Pyruvate		Formate	
	After 20 min.	After 40 min.	After 10 min.	After 40 min.
Inoculum	42.1	29.1	—	—
Early exponential phase	28.5	40.2	—	—
Aminopterin-treated culture	7.7	13.7	50.0	42.0
Control culture	30.4	53.7	72.0	64.1

Table 3. *Inhibition by aminopterin of the phosphoroclastic activity of homogenates of A. aerogenes cells*

The experimental system was as described in Table 1, the cell suspension being replaced by an alumina homogenate (10mg. dry wt. of organisms/ml.) of cells from a 16 hr. static culture of *A. aerogenes*. Residual pyruvate and acetylmethylcarbinol were determined as described in the Materials and Methods section when the evolution of H_2 ceased.

Additions	Rate of H_2 evolution from pyruvate (μ l./hr./mg. dry wt. of organisms)		Total H_2 evolved (μ moles)	Residual pyruvate (μ mole)	Acetylmethylcarbinol content (μ mole)
	At 20 min.	At 60 min.			
None	35	134	9.6	0.15	0.075
Aminopterin (3.4 μ moles)	0	74	9.6	0.31	0.073

Table 4. *Restoration of the phosphoroclastic breakdown of pyruvate in cells and homogenates of aminopterin-treated A. aerogenes by an extract of untreated cells*

The cultures were grown anaerobically and the bacteria harvested after 20 hr. at 37°, washed and resuspended in 0.15 M-NaCl. AM cells were from the culture that was treated with aminopterin (0.126 μ mole/ml.). A portion of the control cell suspension (19.2 mg. dry wt. of organisms/ml.) was heated at 100° for 5 min., diluted with an equal volume of 0.15 M-NaCl and centrifuged at 105400g for 1 hr. (Spinco model L centrifuge, rotor no. 40) at 5°; the clear supernatant solution (144.2 μ g. of N/ml.) was used as 'boiled cell extract'. For the manometric measurements (37°; gas phase N_2) each flask contained 75 μ moles of phosphate buffer, pH 7.0, the cell suspension (5 mg. dry wt. of organisms) or homogenate (0.5 ml.), 10 μ moles of pyruvate in the side arm and 5 N-NaOH (0.2 ml.) in the centre well. Additions of the boiled cell extract (0.5 ml.) and aminopterin (9 μ moles) were made as indicated, and the total fluid volume was adjusted to 2.2 ml. with water. The alumina homogenates (see the Materials and Methods section) were diluted to contain the equivalent of 20 mg. dry wt. of organisms/ml.

		Maximum rate of H_2 evolution (μ l./hr./mg. dry wt. of organisms)			
		None	Boiled cell extract	Aminopterin	Aminopterin + boiled cell extract
Source of enzyme activity					
Control cells		33.4	39.6	23.3	39.6
AM cells		5.6	28.7	—	—
Homogenate of control cells		41.0	38.5	—	—
Homogenate of AM cells		7.3	36.9	—	—

Table 5. *Formate-pyruvate-exchange activities of extracts of cells from control and aminopterin-treated cultures of A. aerogenes*

Cells were harvested after 19–20 hr. at 37° from either static cultures with air as the initial gas phase (Expts. 3, 7 and 13) or from anaerobic ($N_2 + CO_2$, 95:5) cultures (Expt. 14). Extracts prepared by grinding the cells with alumina (see the Materials and Methods section) were centrifuged for 1 hr. at 105400g before use. Exchange activity was measured by the incorporation of [^{14}C]formate into the carboxyl group of pyruvate (see the Materials and Methods section). In each of the aminopterin-treated cultures, the antagonist (0.12 μ mole/ml.) was added in the early exponential phase at a cell density 50–75 μ g. dry wt. of organisms/ml.

Expt. no.	Source of extract	Protein content of extract (mg./ml.)	Period of incubation at 37° (hr.)	Sp. activity of pyruvate 2,4-dinitrophenylhydrazone (counts/min./ μ mole)
3	Control cells	6.8	1.25	1260
	AM cells	6.2	1.25	36
7	Control cells	5.6	1.0	780
	AM cells	5.3	1.0	14
13	Control cells	5.2	1.0	850
	AM cells	5.3	1.0	0
14	Control cells	7.0	2.0	1800
	AM cells	4.2	2.0	84

Table 6. *Inhibition of formate-pyruvate-exchange activity in cell-free extracts of A. aerogenes by aminopterin*

The cells were grown in the chemically defined medium in static (Expt. 25) and anaerobic (Expt. 31) cultures, and anaerobically in peptone-water (Expt. 33). Extracts were prepared by grinding with alumina (Expts. 31 and 33) and in the Hughes press (Expt. 25). Formate-pyruvate-exchange activity was determined by the incorporation of [^{14}C]formate into the carboxyl group of pyruvate, as described in the Materials and Methods section.

Concn. of aminopterin (mM)	Sp. activity (counts/min./ μmole) of pyruvate 2,4-dinitrophenylhydrazone		
	Expt. 25	Expt. 31	Expt. 33
2.2	—	33.6	52.8
1.8	52.4	—	—
1.1	—	42.0	133.2
0.9	73.6	—	—
0.55	—	—	258.4
0.275	—	—	626.2
0.055	—	—	848.8
0.0	587.5	756.8	936.8

uptake (about one-thirtieth of that with pyruvate as substrate) occurred at pH 5.6, and at pH 7.4 the compound inhibited both endogenous oxygen consumption and the oxidation of pyruvate. Anaerobically, 3-methyl-2-oxobutanoate was metabolized slowly with the production of carbon dioxide, but not hydrogen; this reaction also had its maximum rate at about pH 6, and was inhibited completely at pH 7.4. Neither the aerobic nor the anaerobic metabolism was affected by aminopterin. In addition, no exchange occurred between formate and the carboxyl group of 3-methyl-2-oxobutanoate in the presence of extracts that were capable of catalysing the formate-pyruvate exchange, even when supplemented with excess of cysteine (50 μmoles) and CoA (20 $\mu\text{g.}$), ATP (1.5 μmoles), NAD (0.6 μmole), folic acid (1 μmole) and tetrahydrofolic acid (1 μmole).

DISCUSSION

As aminopterin does not inhibit either the synthesis or the utilization of alanine and valine by *A. aerogenes*, it appears that these amino acids, which accumulate in aminopterin-treated cultures of this organism (Webb, 1958), must arise as secondary products from pyruvate and 3-methyl-2-oxobutanoate. Both of these keto acids accumulate in the inhibited cultures; the former, which is the precursor of the latter (Wixom, 1965), is the primary intermediate to accumulate. This accumulation leads to increased concentrations of other metabolites that are derived also from pyruvate

(e.g. lactate, acetylmethylcarbinol, butane-2,3-diol). The production of acetylmethylcarbinol increases disproportionately with pyruvate concentration (Strecker & Ochoa, 1954) and probably this is true also for the valine precursor, 3-methyl-2-oxobutanoate, since α -acetolactate is a common intermediate in both syntheses (e.g. Wixom, 1965).

3-Methyl-2-oxobutanoate is not metabolized by *A. aerogenes* through a phosphoroclastic series of reactions, and no exchange occurs between its carboxyl group and formate in cell-free extracts. In this respect, extracts of *A. aerogenes* differ from those of *Micrococcus lactilyticus*, which catalyse a slow exchange between formate and 3-methyl-2-oxobutanoate as well as α -oxobutyrate and α -oxoglutarate (McCormick, Ordal & Whiteley, 1962a).

Aerobic pathways of pyruvate metabolism in *A. aerogenes* are unaffected by aminopterin. Static cultures of this organism, however, are deficient in oxygen and, particularly during the exponential phase, are essentially anaerobic (e.g. Dagley, Dawes & Morrison, 1950). Under these conditions one important pathway of pyruvate metabolism is by phosphoroclastic breakdown to acetate and formate, the latter being degraded further to hydrogen and carbon dioxide by the formate hydrogenlyase system. Formate hydrogenlyase is not affected significantly by aminopterin. Hence the evolution of hydrogen from pyruvate provides a convenient measure of phosphoroclastic activity by cells from both control and treated cultures. This activity is decreased significantly after the addition of aminopterin to lag-phase cultures.

Extracts of *A. aerogenes*, from which the particulate component(s) of the formate hydrogenlyase complex has been removed, are unable to complete the anaerobic catabolism of pyruvate to acetate, carbon dioxide and hydrogen, but catalyse an exchange of the carboxyl group of the keto acid with that of formate. This exchange is inhibited by the addition of aminopterin either to growing cultures or to a cell-free system, and thus differs from that of *M. lactilyticus* (McCormick *et al.* 1962a).

The relation of the formate-pyruvate exchange to the phosphoroclastic system is uncertain, although the inhibition of these activities in *A. aerogenes* suggests that at least one reaction may be common to both. Chin, Krampitz & Novelli (1957) assumed that occurrence of the exchange reaction in *E. coli* demonstrated the reversibility of the phosphoroclastic reaction, although previously Strecker, Wood & Krampitz (1950) showed that the two enzyme systems differed in stability. In *M. lactilyticus*, formate exchange takes place only under conditions where the phosphoroclastic reaction occurs, but is not directly proportional to pyruvate degradation (McCormick *et al.* 1962a,b).

In this strict anaerobe, which lacks formate hydrogenlyase, pyruvate degradation is controlled by pH, the products in acid and alkaline environments being respectively acetyl phosphate and formate, and acetate, carbon dioxide and hydrogen (Novelli, 1955). In both of these reactions, which, in common with the phosphoroclastic systems of other bacteria (e.g. Deibel & Niven, 1964), require thiamine pyrophosphate, specific exchange occurs between the C₁ compound and the carboxyl group of pyruvate. Whiteley & McCormick (1963) have proposed a sequence of reactions for the alkaline degradation of pyruvate, the initial steps of which are as follows:

where TPP represents thiamine pyrophosphate.

The authors consider that in such a scheme the unidentified labile cofactors that are necessary for formate exchange (Strecker, 1951; Novelli, Gest & Krampitz, 1954; Asnis, Fritz & Glick, 1956; Wood & O'Kane, 1964; McCormick *et al.* 1962b) may be required for the interconversion of 'C₁' and formate.

The possibility that a folinic acid derivative forms the primary acceptor of the C₁ unit has been suggested by Chin *et al.* (1957) and Wood & O'Kane (1964), although Oster & Wood (1964) consider that any stimulation of exchange activity by tetrahydrofolic acid is due to the maintenance of the necessary oxidation-reduction potential, and is unrelated to coenzyme function. Also, in *A. aerogenes*, as in *M. lactilyticus* (McCormick *et al.* 1962b), tetrahydrofolic acid does not stimulate formate exchange. Although this could indicate a requirement for a different form of the coenzyme, as has been found for other folinic acid-dependent reactions in *A. aerogenes* (Jones, Guest & Woods, 1961; Morningstar & Kisliuk, 1965), the direct inhibition by aminopterin of the exchange and phosphoroclastic activities of this organism cannot be explained by 'folic acid antagonism'. Such antagonism would prevent the synthesis, but not the function, of the reduced coenzymes (e.g. Guest & Woods, 1962). It is therefore probable that the sensitivity of these enzyme systems to aminopterin is due to the inactivation or displacement of an unidentified cofactor that is necessary for both exchange and phosphoroclastic activities (e.g. Wood & O'Kane, 1964; McCormick *et al.* 1962b) and that is present in heat-treated extracts of normal cells.

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