STUDIES ON THE MECHANISM OF ACETATE OXIDATION BY BACTERIA

VI. Comparative Patterns of Acetate Oxidation by Citrate-Grown and Acetate-Grown Aerobacter aerogenes

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INTRODUCTION

In paper IV of this series (Ajl and Wong, 1951), it was shown that citrate-grown *Aerobacter aerogenes* may utilize simultaneously a tri- and dicarboxylic acid cycle for acetic acid oxidation. From adaptation data, however, it appeared that acetate-adapted *Aerobacter* behaves more like *Escherichia coli; i.e.*, breaks down acetate *via* a pathway best reconciled with a Knoop-Thunberg condensation mechanism (Ajl, 1950; Ajl and Kamen, 1951). The adaptation experiments revealed that whereas citrate-grown cells adapted themselves to oxidize acetate rapidly, the reverse was not altogether true; *i.e.*, acetate-grown *Aerobacter* was not adapted to oxidize citrate rapidly. Further, acetate-grown *Aerobacter* showed a heightened activity on succinate, fumarate, malate, oxalacetate, and pyruvate, but not on α-ketoglutarate, *cis*-aconitate, or citrate. Similar results were obtained with *E. coli* (Ajl, 1950; Ajl and Kamen, 1951). The suggestion was put forth (Ajl and Wong, 1951), therefore, that *A. aerogenes*, when grown on acetic acid, may oxidize the C_2-fatty acid in a manner similar to that of *E. coli*. The present communication confirms this finding.

In this paper, data obtained on the simultaneous oxidation by acetate-grown *A. aerogenes* of C^{14}-labeled acetate and unlabeled citrate, *cis*-aconitate, α-ketoglutarate, succinate, fumarate, and malate, singly or in combination, are presented and compared with data obtained previously using citrate-grown *Aerobacter* and acetate-adapted *E. coli*. It is shown that the data with acetate-grown and citrate-grown *Aerobacter* are diametrically opposed; *e.g.*, while a conventional tricarboxylic acid cycle may occur in citrate-grown cells, it does not appear to be a major mode of acetate oxidation in acetate-adapted *Aerobacter*. Further, the data with acetate-grown cells are completely analogous to those obtained with acetate-adapted *E. coli*.

Methods

Aerobacter aerogenes (American Type Culture Collection No. 8308) was adapted to utilize acetate rapidly by growing the cells on a medium containing 1.5 per cent

anhydrous sodium acetate, 0.8 per cent KH_2PO_4, 0.4 per cent $(NH_4)_2SO_4$, 0.07 per cent tryptone, and 20 per cent tap water, at an initial pH of 7.0. The cells were Sharples-centrifuged after 48 hours' aeration at 30°C. Endogenous activity was reduced by aerating the cells at room temperature for several hours in phosphate buffer.

Respiration was measured in 30 ml. Warburg vessels. Oxygen uptake was measured at 30°C. with NaOH in the center well to absorb carbon dioxide. Distillation, isolation, and chromatographic procedures were those previously described (Ajl and Wong, 1951; Ajl and Kamen, 1951).

All the radioactive assays were made using a conventional end window G-M tube counter connected to a Tracerlab autoscaler. Solutions were pipetted on recessed stainless steel discs after neutralization. Solids such as protein, cell material, and $BaCO_3$ were slurried in ethyl alcohol and plated evenly on discs. Drying was conducted under an infrared lamp. Appropriate corrections for self-absorption and carbon content were applied.

The C^{14}-methyl-labeled sodium acetate was obtained from Tracerlab, Inc., Boston, Mass., and exhibited an initial activity of $\sim 4 \times 10^5$ C.P.M./mg., as assayed with the geometry used. Carboxyl-labeled acetate was synthesized by the method of Ruben, Allen, and Nahinsky (1942).

EXPERIMENTAL DATA

Oxidation of 2-C^{14} Acetate.—The distribution of labeled carbon after oxidation of methyl-labeled acetate by acetate-grown *A. aerogenes* is shown in Table I. The major datum in this table is the 1.92-fold dilution of acetate resulting from turnover with endogenous substances. Similar results were obtained with acetate-grown *E. coli* (Ajl and Kamen, 1951) where acetic acid appears to be oxidized by a Knoop-Thunberg condensation mechanism. Citrate-grown *Aerobacter*, however, showed little or no dilution of acetate as would be expected on the basis of the conventional tricarboxylic acid cycle (Ajl and Wong, 1951).

Preliminary experiments of a semiquantitative nature were performed, in each of which methyl-labeled acetate was partially oxidized in the presence of unlabeled citrate, α-ketoglutarate, or succinate (Table II).

It was found that under conditions in which all three substrates were available to the bacteria, as evidenced by oxidative metabolism, acetate carbon was recovered only in succinate and not in α-ketoglutarate. After 6 hours' incubation, some activity was found in citrate. These exploratory experiments revealed that oxidation of 108.5 μM of acetate, in the presence of either 100 μM

$$\frac{\text{Final activity of succinate}}{\text{Average specific activity of acetate}} \text{ or } \frac{6{,}360}{\frac{371 + 270}{2}} = \frac{6{,}360}{320} = 19.9$$

citrate, α-ketoglutarate, or succinate, trapped ~20 μM of acetate carbon in succinate, while less than 0.02 μM of acetate carbon were trapped in α-ketoglutarate and less than 0.5 μM of acetate carbon in citrate. In the absence of carrier, little or no activity was found in the ether-soluble fraction which would presumably contain all of the tri- and dicarboxylic acids formed during the

TABLE I

Distribution of Labeled Carbon after Oxidation of Methyl-Labeled Acetate by Acetate-Grown Aerobacter aerogenes

Fraction	Chemical data		Total C^{14}		Specific activity		Average specific activity
	Initial	Final	Initial	Final	Initial	Final	
	μM	μM	C.P.M.	C.P.M.	C.P.M. per μM	C.P.M. per μM	C.P.M. per μM
Acetate	108.5	88	41,075	17,075	371	193	282
Cells				1,600			
Carbonate		160	0	10,120	0	632	316
Non-volatile ether-soluble fraction*				600			
Non-volatile ether-insoluble fraction				100			

The complete system consisted of 1 ml. 0.2 M phosphate buffer, pH 7.0; 1 ml. of a 10 per cent suspension of freshly harvested (acetate-grown) *A. aerogenes;* 2-C^{14}–acetate as indicated; alkali in center well and distilled water to volume. Aerobic. Temperature 30°C. Time of incubation 6 hours.

* This fraction would contain all the Krebs cycle intermediates formed during the course of the reaction.

TABLE II

Oxidation of Methyl-Labeled Acetate by Acetate-Grown Aerobacter aerogenes in the Presence of Either Citrate, α-Ketoglutarate, or Succinate

Experiment No.	Substrate	Carrier	Distribution of activity						Specific activity of residual acetate*
			Cells	Carbonate	Residual acetate	Succinate	α-Ketoglutarate	Citrate	
		C.P.M.	C.P.M.	C.P.M.	C.P.M.	C.P.M.	C.P.M.	C.P.M.	C.P.M. per μM
1	2-C^{14}–acetate	—	1,600	10,120	17,075				193
2	"	Succinate	512		23,475	6,360			270
3	"	α-Ketoglutarate	1,280		31,500		4		350
		Succinate‡				710			
4	"	Citrate	1,408	9,978	22,300			100	173
		Succinate				88			

Total volume of reactants 10 ml. 1 ml. of a 10 per cent suspension of *A. aerogenes;* 1 ml. of 0.2 M phosphate buffer, pH 7.0; carriers in 100 μM concentrations; 108 μM of 2-C^{14}–acetate per flask; alkali in center well and distilled water to volume. Aerobic. Temperature 30°C. Time of incubation 6 hours.

* Initial specific activity of acetate; 371 C.P.M. per μM.

‡ Underlined substances in "carrier" column were formed during the reaction and were not initially added as carriers.

course of the reaction. These results are diametrically opposed to those obtained with citrate-grown cells in which approximately the same amount of acetate carbon was trapped in each member of the tricarboxylic acid cycle (Ajl and

Wong, 1951). Further, the activity in the case of citrate-grown *Aerobacter* could be trapped by adding carrier either in the beginning or at the end of the incubation period. This is not the case with acetate-grown *Aerobacter* or acetate-adapted *E. coli* (Table III).

From the data in Table II, it can be calculated that only 0.5 μM of acetate carbon was trapped in citrate. It may be argued from the adaptation data presented in an earlier paper (Ajl and Wong, 1951), that citrate did not become appreciably active because it did not enter the acetate-adapted cells. This is not the case. Citrate, to be sure, is not oxidatively metabolized in the first 30 minutes of incubation (Ajl and Wong, 1951). The experiment in Table II, however, was carried out for 6 hours and appreciable citrate is oxidized in that period of time (Fig. 1).

TABLE III

The Trapping of Acetate Carbon by Tricarboxylic Acid Cycle Intermediates

Oxidation of acetate	Activity of ether-soluble fraction, C.P.M./min.		
	Acetate-grown		Citrate-grown *A. aerogenes*
	E. coli	*A. aerogenes*	
Without carrier	568	620	2,000*
With carrier succinate	7,480	6,400	2,460*
With carrier α-ketoglutarate	600	730	2,500*
With carrier citrate	703	600	1,960*

In this experiment the total activity of the initial acetate was 10,000 C.P.M.

* Note that with citrate-grown cells it makes little difference whether or not carrier is added.

In order to show in more detail that citrate and α-ketoglutarate are probably not involved in acetate oxidation by acetate-grown *Aerobacter*, a relatively short time quantitative experiment using carboxyl-labeled acetate was performed with carriers indicated (Table IV).

It can be shown that, assuming a Knoop-Thunberg mechanism for acetate oxidation by acetate-grown *Aerobacter*, all the data in Table IV can be correlated satisfactorily, as a result of which a prediction can be made for O_2-uptake which agrees closely with that observed.

Consider first the isotopic dilution to be expected. No dilution will arise if a citric acid cycle is operative, except from endogenous sources. If one excludes a citric acid cycle because of the absence of acetate carbon in α-ketoglutarate and citrate, then the dilution calculated solely on the basis of acetate arising from the postulated degradation of succinate, α-ketoglutarate, and citrate can be shown to agree quantitatively with that found. Thus, from the average specific C^{14} content of acetate (1145

C.P.M. per μM) and the total C^{14} content in succinate (8640 C.P.M.), approximately 8 μM $\left(\frac{8640}{1145}\right)$ of acetate carboxyl carbon could have been condensed to succinate yielding 4 μM of succinate. From the data in Table IV, it is clear that 10 μM of succinate, 8 μM of α-ketoglutarate, and 4 μM of citrate disappeared. Adding to this 4 μM of succinate additional to compensate for succinate assumed to be formed, the total O_2-uptake expected would be 43 μM. This latter value was obtained in the following manner: 8 μM of acetate condensing to succinate would take up 4 μM of O_2; 10 μM of succinate was oxidized taking up 15 μM of O_2; 8 μM of α-ketoglutarate disappeared

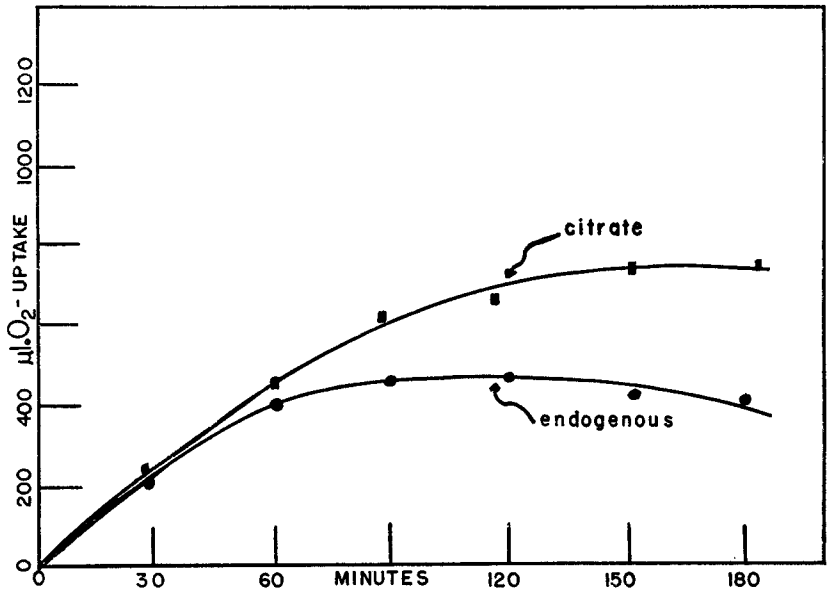

FIG. 1. Oxidation of citrate by acetate-grown *Aerobacter*. Total volume of reactants 2.3 ml. Each vessel contained 0.5 ml. 0.2 M phosphate buffer, pH 7.0. NaOH in center well and distilled water to volume. To experimental cups 50 μM of sodium citrate was added. Aerobic. Temperature 30°C.

consuming 16 μM of O_2, assuming that the oxidation of the C_5-keto acid proceeded *via* succinate; and 4 μM of citrate was oxidized taking up 2 μM of O_2, if we assume that the citrate was first broken down to acetate and oxalacetate and the latter was oxidized to acetate with only 0.5 mole of oxygen per mole of oxalacetate. In addition, the 4 μM of succinate which was formed from radioactive acetate was oxidized, consuming an additional 6 μM of O_2. Thus, the over-all total adds up to 43 μM of O_2-uptake. A total of 30 μM acetate, essentially unlabeled, should have arisen from the oxidation of citrate, α-ketoglutarate, and succinate. The figure of 30 was arrived at as a result of the following calculations: 10 μM from succinate, 8 μM from α-ketoglutarate, 8 μM

from citrate (by a direct split of 4 μM citrate, 4 μM of oxalacetate and 4 μM of acetate would form; the formed oxalacetate under aerobic conditions would in turn yield 4

TABLE IV

Quantitative Data of Acetate Oxidation by Acetate-Grown Aerobacter

	Fraction	Amount	C^{14} content	Specific activity
		μM	C.P.M.	C.P.M. per μM
Initial conditions	Acetate	44	69,150	1,571
	Succinate	50	0	
	α-Ketoglutarate	50	0	
	Citrate	55	0	
	Carbonate			
Final conditions	Acetate	57	40,975	719
	Succinate	40	8,640	216
	Methine carbons		—	—
	Carboxyl carbons		~8,000	
	α-Ketoglutarate	42	65	3
	Citrate	51	45	<1
	Carbonate		17,008	
	Cells		1,401	

Total volume of reactants 10 ml. Each vessel contained carboxyl-labeled acetate and carriers as indicated. In addition each flask contained 1 ml. of 0.2 M phosphate buffer, pH 7.0; 1 ml. of a 20 per cent suspension of *A. aerogenes;* 3 ml. NaOH in center well, and distilled water to volume. Temperature 30°C. Time, 90 minutes.

TABLE V

Oxidation of Methyl-Labeled Acetate in the Presence of α-Ketoglutarate and Cis-Aconitate

Incubation mixture	Products reisolated	Total counts
		C.P.M.
2-C^{14}-acetate; *cis*-aconitate; α-ketoglutarate	2-C^{14}-acetate	46,750
	cis-Aconitate	7
	Citrate*	3
	α-Ketoglutarate	23
	Succinate*	674

Total volume of reactants 10 ml. The Warburg vessel contained 108 μM of 2-C^{14}-acetate (82,000 C.P.M.); 100 μM each of α-ketoglutarate and *cis*-aconitate; 1 ml. of 0.2 M phosphate buffer, pH 7.0; 1 ml. of a 10 per cent suspension of acetate-grown *A. aerogenes;* NaOH in center well and distilled water to volume. Aerobic. Temperature 30°C. Time, 3 hours.

* Formed during the course of the reaction.

μM of acetic acid, thus making a total of 8 μM of the C_2-fatty acid), and 4 μM of acetate from the formed succinate. Adding this to the original 44 μM of acetate, there would

result a total of 74 μM of acetate accumulated. As a first approximation, there should be subtracted the 8 μM lost by condensation to succinate, as well as $\sim$1 μM appearing in the acid-insoluble cell material and hence metabolized without involving appreciable oxygen uptake. Hence, if no acetate were oxidized, accumulation of 65 μM acetate would be expected (74 minus 9). 57 μM was found. The difference, 8 μM, could be ascribed to oxidation of acetate resulting in CO_2 and water. This would require 16 μM additional O_2-uptake, giving a total of 59 μM or 1321 μl.; 1300 μl. was actually observed. On this basis the dilution of acetate could be calculated precisely enough by assuming that 16 μM (8 + 8) of labeled acetate was removed and replaced by the

TABLE VI

Oxidation of Carboxyl-Labeled Acetate in Presence of Succinate, Fumarate, or Malate

Experiment No.	Activity in ether-soluble fraction	Carrier	Activity associated with carrier
	C.P.M.		C.P.M.
1	20,875	Succinate	19,300
2	21,900	Succinate	9,400
		Fumarate	6,840
		Malate	7,300
3	13,900	Fumarate	5,410
		Malate	8,120
4	800	No carrier	

Each reaction vessel contained 100 μM of labeled acetate (156,000 C.P.M.); 1 ml. of 0.2 M phosphate buffer, pH 7.0; 1 ml. of a 10 per cent suspension of acetate-adapted *A. aerogenes;* alkali in center well and distilled water to 10 ml. Carriers were added in 50 μM concentrations. Time, 3 hours. Temperature 30°C. Aerobic.

43 μM of unlabeled acetate derived from succinate, α-ketoglutarate, and citrate degradation. The final specific activity could not be less than $1{,}571\left(\frac{43}{57}\right)$ or 1,185 C.P.M. per μM. The observed value was 719 C.P.M. per μM. It should be noted that on the basis of this calculation, 8 μM of acetate "were condensed" simultaneously with oxidation of 8 μM, a ratio of oxidized to condensed acetate of exactly *one*, which is precisely what would be expected if a Knoop-Thunberg mechanism were operating.

It would be of some interest to calculate the theoretical oxygen uptake on the basis of citrate breaking down *via* α-ketoglutarate. Each mole of citrate would thus require 2.5 moles of O_2 to yield 1 mole of acetate. Since in the experiments just described 4 μM of citrate disappeared, that would require 10 μM of O_2-uptake instead of 2 μM if citrate were broken down directly to acetate and oxalacetate by acetate-grown *Aerobacter*. Thus, instead of an O_2-uptake of 1,300 μl., 1,404 μl. should have been observed, a figure considerably higher than the theoretical (1,321 μl.).

One experiment was performed in which methyl-labeled acetate was oxidized in the presence of α-ketoglutarate and *cis*-aconitate (Table V). The results obtained are qualitatively identical with those presented in Table IV. It is of interest that two compounds, not originally present, were formed in this reaction; *e.g.*, succinate and citrate. The citrate formed had no activity whatsoever, whereas the C_4-dicarboxylic acid contained almost all the activity; *i.e.*, ~674 C.P.M.

Thus far all the data obtained with acetate-grown *Aerobacter* agree well with the data for acetate-adapted *E. coli*. A difference between the two organisms can, however, be detected. In the case of *E. coli* (Ajl and Kamen, 1951), a high endogenous production of succinate from more oxidized C_4-dicarboxylic acids always took place in our Warburg cups. This resulted in entrapment of most of the activity in succinate, regardless of whether fumarate, malate, or oxalacetate was the carrier. This is not the case with acetate-adapted *Aerobacter*. The activity is always associated with the carrier and no significant back reduction to succinate takes place (Table VI). There is, therefore, complete equilibration with each C_4-dicarboxylic acid added.

DISCUSSION

The experimental findings in the previous section indicate that the same organism may possess two mechanisms for the oxidation of the same substrate, depending upon the carbon source it uses for growth. Irrespective of whether the incorporation of acetate carbon into succinate, fumarate, and malate (but not into α-ketoglutarate and citrate) indicates the operation of a dicarboxylic acid cycle, and the incorporation of acetate carbon into all the tri- and dicarboxylic acids signifies the operation of a tricarboxylic acid cycle, acetate-grown and citrate-grown *Aerobacter* appear to possess different mechanisms for the oxidation of the same substrate. Acetate-adapted *Aerobacter* behaves very much like *E. coli* with respect to the oxidation of the C_2-fatty acid, whereas citrate-grown *Aerobacter* follows *M. lysodeikticus* in its pattern of acetate oxidation. Now the question is this: What evidence is there to support the view that acetate-grown *Aerobacter* possesses a dicarboxylic acid cycle, whereas citrate-grown cells oxidize acetic acid mainly *via* the conventional tricarboxylic acid cycle? Evidence supporting the latter has been published elsewhere (Ajl and Wong, 1951) and need not be repeated here. The data in this paper which indicates that a Knoop-Thunberg condensation *may be* the mechanism which is involved in acetate oxidation by acetate-grown *A. aerogenes* are (1) the complete failure of α-ketoglutarate and citrate to trap acetate carbon under conditions in which acetate carbon is oxidized and trapped quantitatively in succinate, fumarate, and malate; (2) as can be calculated from the data in Table IV, exactly the same number of micromoles of acetate was condensed to succinate as was oxidized to CO_2 and water; and (3) the observed O_2-uptake

agrees well with the theoretical, if it is assumed that citrate is broken down directly to acetate and oxalacetate and not *via* α-ketoglutarate, as would be expected on the basis of a Krebs' cycle.

Based upon these findings and those previously published (Ajl and Wong, 1951; Ajl, 1950; Ajl and Kamen, 1951), the following may be considered to be the terminal oxidation pattern of *A. aerogenes*. Glucose- and glycerol-grown cells will oxidize these and other products to the stage of acetate and stop there. It has been shown (Ajl, 1950) that glucose-grown cells do not oxidize acetate and that acetate accumulates during glucose oxidation. When the same organism is grown on acetate, it will oxidize the C_2-acid chiefly by a mechanism best reconciled with a Knoop-Thunberg condensation reaction as shown by the isotope and adaptation data. During growth on acetate, the organism apparently does not elaborate the enzymes involved in citrate breakdown, through *cis*-aconitate, α-ketoglutarate, etc. However if citrate constitutes the source of carbon for growth, the organism does appear to elaborate the enzymes involved for the conventional pattern of citrate oxidation and may, therefore, gear the acetate oxidation through citrate and the conventional tricarboxylic acid cycle.

SUMMARY

The data presented in this paper indicate operation of different mechanisms for acetate oxidation by *A. aerogenes*, depending on the carbon source used for growth. The mechanism for citrate-grown cells appears to involve a conventional citric acid cycle, whereas acetate-grown cells appear to incorporate acetate carbon more readily *via* a dicarboxylic acid cycle.

Note added to Galley Proof.—While this paper was in press a letter to the editors appeared by Dagley, Morrison, and Dawes (*Arch. Biochem. and Biophys.*, 1951, **32,** 231) implicating a dicarboxylic acid cycle in the metabolism of *A. aerogenes*.

BIBLIOGRAPHY

Ajl, S. J., and Wong, D. T. O., 1951, Studies on the mechanism of acetate oxidation by bacteria. IV. Acetate oxidation by citrate-grown *Aerobacter aerogenes* studied with radioactive carbon, *J. Bact.*, **61,** 379.

Ajl, S. J., 1950, Acetic acid oxidation by *Escherichia coli* and *Aerobacter aerogenes*, *J. Bact.*, **59,** 499.

Ajl, S. J., and Kamen, M. D., 1951, Studies on the mechanism of acetate oxidation by *Escherichia coli*, *J. Biol. Chem.*, **189,** 845.

Ruben, S., Allen, M., and Nahinsky, P., 1942, Tracer studies with radioactive carbon. The exchange between acetic acid anhydride and sodium acetate, *J. Am. Chem. Soc.*, **64,** 3050.