INDUCED SYNTHESIS OF TRICARBOXYLIC ACID CYCLE ENZYMES AS CORRELATED WITH THE OXIDATION OF ACETATE AND GLUCOSE BY PASTEURELLA PESTIS[1]

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A preliminary enzymatic analysis of the adaptation of anaerobically grown *Pasteurella pestis*, strain A1122, to an oxidative metabolism has demonstrated the induced synthesis of isocitric dehydrogenase, aconitase, fumarase, and cytochrome (Englesberg *et al.*, 1954*a*,*b*). The present paper will describe the other enzymes involved in the adaptation and present further evidence indicating that the tricarboxylic acid cycle is necessary for the oxidation of acetate and for the "complete" oxidation of glucose by aerobically grown *P. pestis*.

MATERIALS AND METHODS

Pasteurella pestis strain A1122, the medium, cultural conditions, the preparation of enzymatically active cell-free extracts, and the procedures employed for adapting anaerobically grown cells to an oxidative metabolism are as previously described (Englesberg *et al.*, 1954*a*,*b*). Cell-free extracts of aerobically grown cells (aerobic extracts), of anaerobically grown cells (anaerobic extracts), and of anaerobically grown but aerobically adapted cells (aerobic adapted extracts), prepared under the same conditions and of the same age and storage history, are compared as to enzyme activity. Activity is expressed as the number of micromoles of substrate decomposed or product formed per hour per mg N. The nitrogen content of the cell extracts was determined by the micro-Kjeldahl method of Ma and Zuazaga (1942).

Acetate activating enzyme. The initial procedure for the estimation of activity of the acetate activating enzyme was based upon the use of components in the acetate-coenzyme A-adenosine triphosphate reaction, as described by Chou and Lipmann (1952) and by Beinert *et al.* (1953) (see table 1). The components in the system were eventually modified with the elimination of unnecessary cofactors (coenzyme A and cysteine) and KCl and by arriving at the optimum concentration of acetate for the reaction (approximately 240 μM acetate per reaction mixture). Active acetate formation was estimated by the hydroxamic acid method of Lipmann and Tuttle (1945) by incorporating potassium hydroxide neutralized hydroxylamine HCl in the reaction mixture.

Phosphotransacetylase. Phosphotransacetylase activity was measured by the arsenolysis method of Stadtman *et al.* (1951) and is represented as the number of μM of acetyl phosphate decomposed per hour per mg N per 0.18 μM of added coenzyme A. Activity is estimated from the difference between the amount of acetyl phosphate decomposed during the first 15 minutes after the addition of the enzyme in the presence and in the absence of added coenzyme A. This is possible since the amount of coenzyme A present in the extracts is insignificantly small as compared to the amount added and since the phosphotransacetylase activity is proportional to the amount of coenzyme A added over a wide range of concentration.

Oxidative decarboxylation of pyruvate. The rate of pyruvate dissimilation was determined by measuring the amount of CO_2 evolved in an atmosphere of N_2 at 30 C during the first 5 minutes after the addition of substrate. The main chamber of the Warburg vessel contained potassium phosphate buffer, pH 7.4 (66 μM), $MgCl_2$ (10 μM), coenzyme I (10 μM), coenzyme A (0.18 μM), cysteine HCl (20 μM), cocarboxylase (1 μM), and cell extract to a total of 2.5 ml. To one side arm was added 0.1 ml of 4 N H_2SO_4 and

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to the other 0.2 ml of 0.1 M sodium pyruvate. Vessels were also set up to determine bound and endogenous CO_2 production. The products of anaerobic dissimilation of pyruvate under these conditions were determined by allowing the reaction to go for one hour before dumping the sulfuric acid. The supernatant was assayed for residual pyruvate and for lactate, acetate, formate, and ethanol. Similar assays were performed on supernatants obtained from vessels employed in determining endogenous CO_2 production, and the values obtained were subtracted.

Citrate synthesis. The cell extracts were compared as to their ability to synthesize citrate from: (1) acetyl phosphate and oxalacetate (in the presence of an excess of phosphotransacetylase from *Clostridium kluyveri*); (2) acetate and oxalacetate; and (3) pyruvate and oxalacetate. The reaction mixtures were incubated at 30 C in an atmosphere of 95 per cent N_2 and 5 per cent CO_2. Because of the low aconitase activity (Englesberg *et al.*, 1954*b*) insignificant amounts of citrate formed are lost during the course of the experiment. Activity measurements are based upon the amount of citrate synthesized during the first 15 minutes after the addition of the enzyme. Citrate formed was determined by the procedure of Taylor (1953) with certain modifications.

Isocitric dehydrogenase. The activity of this enzyme was determined as previously described (Englesberg *et al.*, 1954*b*).

α-Ketoglutarate dehydrogenase. α-Ketoglutarate dehydrogenase activity was measured by determining the rate of CO_2 production from α-ketoglutarate manometrically in an atmosphere of N_2 at 30 C in the presence of the required cofactors. The procedure is essentially the same as that described for pyruvate except for variation in the cofactors added (see table 6).

Succinic dehydrogenase. The activity of succinic dehydrogenase was determined by following the reduction of sodium 2,6-dichlorobenzenoneindophenol spectrophotometrically at 600 mμ (Repaske *et al.*, 1953) and is based upon the change in optical density during the second 30 second period after the addition of the cell extract. The molar extinction coefficient of 2,6-dichlorobenzenoneindophenol employed was found to be 1.85×10^4 at pH 7.5. The complete system consisted of sodium succinate (30 μM), histidine (30 μM), $MgSO_4$ (4 μM), NaCN (pH 7, 24 μM), sodium 2,6-dichlorobenzenoneindophenol (1.8×10^{-3} per cent), and enzyme preparation in a total volume of 3 ml at a final pH of 7.5. The reaction was started by the addition of enzyme preparation. The rate of endogenous reduction of the dye, which was quite high, was followed separately and subtracted from the rate in the presence of succinate. Water was employed as a blank. To identify the products of this reaction, the oxidation of succinate was followed manometrically, employing oxygen as the hydrogen acceptor. The complete system consisted of phosphate buffer, pH 7 (66 μM), $MgSO_4$ (2 μM), and sodium succinate (20 μM). By the omission of coenzyme I from the system, the oxidation of succinate was stopped at the fumarate level, the extracts themselves being deficient in this cofactor. At the completion of oxygen uptake with succinate, the reaction mixture was deproteinized with sodium tungstate and sulfuric acid and the supernatant extracted with ether for several hours. The ether extract was evaporated to dryness and the residue chromatographed on paper.

Malic dehydrogenase. Malic dehydrogenase activity was determined by measuring the reduction of coenzyme I spectrophotometrically at 340 mμ. The complete system consisted of tris-(hydroxymethyl)aminomethane buffer at pH 7.5 (150 μM), $MnCl_2$ (30 μM), coenzyme I (2 μM), NaCN (pH 7, 50 μM), L-malate (10 μM), and 0.05 to 0.1 ml of cell-free extract to a final volume of 3 ml. The blank contained all of the above except substrate. Activity is based upon the amount of coenzyme I reduced during the second 30 second period after the addition of substrate.

Glyceraldehyde-phosphate dehydrogenase. Glyceraldehyde-phosphate dehydrogenase activity was determined by measuring the reduction of coenzyme I spectrophotometrically at 340 mμ according to the procedure of Cori *et al.* (1945) with modifications. The complete system consisted of potassium phosphate buffer, pH 8.0 (33 μM), coenzyme I (2 μM), cysteine (20 μM), sodium arsenate (50 μM), DL-glyceraldehyde-phosphate (4.8 μM), cell-free extract, and water to a final volume of 3 ml. The blank contained all the above except substrate. Activity is based upon the amount of coenzyme I reduced during the second 30 second period after the addition of substrate.

Phosphofructokinase. Phosphofructokinase ac-

tivity was measured manometrically according to the procedure as described by Colowick and Kalckar (1943). The complete system consisted of bicarbonate buffer (82.5 μM), sodium iodoacetate (6 μM), potassium fluoride (120 μM), fructose-6-phosphate (30 μM), adenosine triphosphate (10 μM), cell extract, and water to yield a final volume of 3.0 ml. A control vessel containing the above ingredients less fructose-6-phosphate was employed as a measure of adenosine triphosphatase activity. The reaction was carried out at 30 C in an atmosphere of 95 per cent N_2 and 5 per cent CO_2. The rate of phosphofructokinase activity, expressed as the number of μM of fructose-6-phosphate esterified per hour per mg N, is based upon the amount of CO_2 evolved during the second 5 minute interval after the addition of substrate less the amount of CO_2 liberated from adenosine triphosphate alone. In all cases this corresponded to the highest rate attained.

Oxalacetate decarboxylase. The complete system consisted of phosphate buffer, pH 7.0 (66 μM), sodium oxalacetate (20 μM), $MgCl_2$ (2 μM), and cell extract in a total volume of 2.6 ml. The reaction was carried out under N_2 at 30 C. The rate of oxalacetate decarboxylation is based upon the amount of CO_2 evolved during the first 5 minutes after the addition of substrate. With the omission of coenzyme I, the CO_2 evolved is essentially that from the decarboxylation of oxalacetate since pyruvate produced is decarboxylated only slowly in the absence of added coenzyme I.

Oxidative production of succinate from fumarate employing fluoride as an inhibitor of succinate oxidation and carbon balance. Oxygen uptake and CO_2 evolution were measured in the Warburg apparatus with fumarate as substrate in the presence and absence of fluoride. Endogenous respiration was measured in a similar fashion, and succinate oxidation with and without fluoride was run as a control. The reaction mixture consisted of 100 μM of phosphate buffer (pH 7.0), 2 ml of cell extract, 2 μM of coenzyme I, 2 μM of adenosine triphosphate, 2 μM of $MgSO_4$, and 20 μM of substrate (when employed) with or without 96 μM of potassium fluoride in a total volume of 3.3 ml. Sulfuric acid was added from the side arm at the point when the rate of oxygen consumed approached the endogenous respiratory level. The contents of the Warburg vessels were washed out, and a limiting amount of sodium tungstate was added to complete the deproteinization. Samples of the supernatants were assayed for residual fumarate and for acetate, lactate, and succinate as indicated below. Assays were performed on the supernatants obtained from oxidation of fumarate, fumarate plus fluoride, and from the endogenous respiration with and without fluoride. The values obtained from the latter were subtracted from the corresponding values obtained from the oxidation of substrate.

Chemical analysis. Acetic acid was determined by microtitration of a steam distillate of a sulfuric acid deproteinized supernatant. In later experiments the micro-low temperature vacuum distillation method of Grant (1946) was employed. No formic acid was detected in the distillates as indicated by the sensitive colorimetric procedure of Grant (1948). Pyruvate was determined by the method of Friedemann and Haugen (1943); ethanol, by the microdiffusion method of Winnick (1942); and lactic acid, by the method of Barker and Summerson (1941) as modified by Neish (1950). Active acetate formation or acetyl phosphate decomposition was determined by the hydroxamic acid method of Lipmann and Tuttle (1945). Succinic acid was determined by the succinic oxidase method (Umbreit *et al.*, 1949).

Paper chromatography was employed to identify fumarate and malate as products of succinate oxidation in the absence of coenzyme I. The following solvent mixtures were employed: (1) ether (peroxide free) (70 ml), benzene (30 ml), H_2O (10 ml), formic acid (4 ml)[3]; (2) butanol, formic acid, water mixture (Lugg and Overell, 1948); and (3) phenol, water, formic acid mixture (Opieńska-Blauth *et al.*, 1951). Whatman no. 1 paper was employed either in horizontal or ascending chromatography, and pie plates

[3] The mixture was shaken in a separatory funnel and the water layer discarded. An additional 1 ml of formic acid was then added. Before being placed in the solvent, the paper was saturated with water vapor by being placed in a closed container over water for several hours. Except for the reduced formic acid concentration, the solvent mixture is essentially that described by Adelberg *et al.* (1951). The R_f for the common acids is given elsewhere (Englesberg, 1950). We would like to thank Dr. Adelberg for bringing this method to our attention before publication.

TABLE 1

Active acetate synthesis

The basic system consisted of tris(hydroxymethyl)aminomethane buffer of pH 7.5 (150 μM), KCl (60 μM), $MgCl_2$ (10 μM), hydroxylamine, pH 7 (800 μM), potassium adenosine triphosphate (10 μM), coenzyme A (0.18 μM), cysteine HCl (20 μM), potassium acetate (10 μM), and cell-free extract in a total volume of 2 ml. The mixture without the cell-free extract was incubated for 5 minutes at 30 C. The extract was then added, and incubation was continued for an additional 30 minutes.

	μM Active Acetate Formed per Hr per Mg N	
	Aerobic extract no. 24	Anaerobic extract no. 25
Complete system	4.51	4.34
Less KCl	4.63	4.37
Less KCl, coenzyme A, and cysteine	4.40	5.66
Less KCl, coenzyme A, and adenosine triphosphate	0.20	0.93
Less KCl, coenzyme A, and $MgCl_2$	1.18	1.04

were employed in the former procedure. The papers were sprayed with an ethanol solution of bromcresol green and with a dilute solution of $KMnO_4$, the latter to further identify fumaric acid. The spots were identified by running the unknown alone and with the addition of each of the possible compounds that might be present. The amount of each compound present was estimated by means of a standard straight line curve, previously obtained for each substance, relating spot area to spot content.

Coenzyme I, cocarboxylase, cytochrome c, coenzyme A (70 to 75 per cent pure), sodium pyruvate, α-ketoglutarate, and fructose-6-phosphate (di-barium salt) were obtained from Nutritional Biochemicals. Acetyl phosphate was obtained from Dr. H. Barker and from Schwarz Laboratories. Potassium adenosine triphosphate was obtained from Sigma Chemical Company. Acetone dried *C. kluyveri*, generously supplied by Dr. H. Barker, was employed in preparing a rough phosphotransacetylase. The acetone dried cells were ground (5 per cent solution) in 0.01 M potassium phosphate buffer at pH 6.6. The suspension was allowed to stand for 1½ hours at room temperature. The debris was spun down at 10,000 rpm, and the supernatant removed and

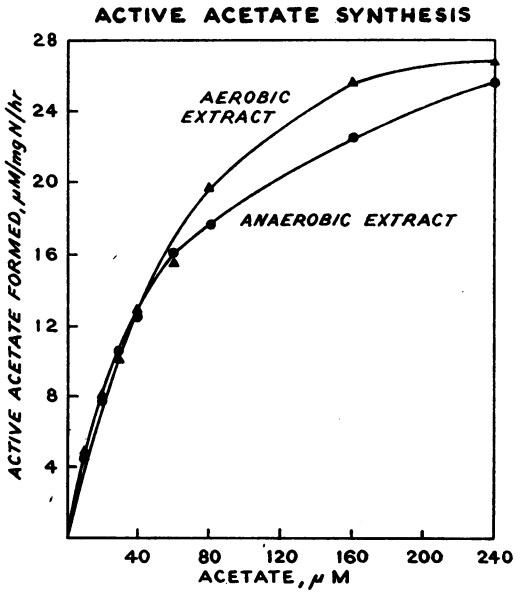

Figure 1. A comparison of the active acetate forming activity of cell-free extracts of aerobically and anaerobically grown cells as a function of acetate concentration. The basic system and procedure are as described in table 1.

stored for use in the deep freeze.[4] 2,6-Dichlorobenzenoneindophenol was obtained from Mathieson Chemical Company. DL-Glyceraldehyde phosphate was prepared from the dioxane addition compound of DL-glyceraldehyde L-bromide 3-phosphoric acid, which was generously supplied by Dr. D. L. Macdonald.

RESULTS

Active acetate synthesis. The initial components employed in this assay are summarized in table 1. The activity of extracts of aerobically and anaerobically grown cells under these conditions appears to be essentially the same. The addition of KCl, coenzyme A, and cysteine apparently is not required for the reaction, whereas adenosine triphosphate and $MgCl_2$ are. Increasing the concentration of adenosine triphosphate and $MgCl_2$ had no further stimulatory effect on the rate of active acetate formation, whereas increases in the concentration of acetate caused a proportionate increase in rate. Figure 1 demonstrates that the amount of active acetate synthesized continues to increase over a wide range

[4] This procedure was suggested to us by Dr. H. Barker, to whom we express our gratitude.

of acetate concentration. The enzyme system of both aerobic and anaerobic cells is saturated with approximately 200 to 240 μM of acetate per 0.5 mg of cell extract N. Employing 240 μM of acetate and doubling the amounts of adenosine triphosphate, $MgCl_2$, coenzyme A, and cysteine cause no further increase in rates of active acetate synthesis. A comparison of several aerobic, anaerobic, and aerobic adapted cell extracts under the optimum conditions thus arrived at indicates no significant difference between the three types of cell extracts with regard to rates of active acetate formation (see table 9). Since the failure to demonstrate an increase in rate of active acetate synthesis with the addition of coenzyme A might be due to the presence of excess coenzyme A in the cell extracts, attempts were made to free the system of this cofactor. Extracts dialyzed against distilled water failed to show a coenzyme A requirement. The cell extracts, treated with acid washed "norite" according to the procedure outlined by Stadtman *et al.* (1951), which was designed to remove coenzyme A, showed no requirement for this factor although a partial requirement for cysteine became apparent. From these results it appears that both aerobically and anaerobically grown cells contain an enzyme in approximately the same concentration, which activates acetate by a coenzyme A independent acetokinase reaction, probably by converting acetate to acetyl phosphate directly. A similar reaction has been described by Rose *et al.* (1954) for *Streptococcus hemolyticus* and *Escherichia coli*.

TABLE 2

Oxidative decarboxylation of pyruvate

The reaction mixture contained potassium phosphate buffer, pH 7.4 (66 μM), pyruvate (20 μM), $MgCl_2$ (10 μM), coenzyme I (10 μM), coenzyme A (0.18 μM), cysteine HCl (20 μM), cocarboxylase (1 μM), and cell extract to a total volume of 2.7 ml. The reaction was carried out under N_2 at 30 C. The rate of pyruvate dissimilation is based upon the amount of CO_2 evolved during the first 5 minutes after addition of pyruvate.

	μM Pyruvate Decomposed per Hr per Mg N		
	Aerobic extract no. 33	Anaerobic extract no. 27	Aerobic adapted extract no. 28
Complete system	21.9 19.8	3.7	25.4
Less coenzyme A and cysteine	3.8		
Less coenzyme A, cysteine, and cocarboxylase	1.9		
Less coenzyme I	4.9		
Less coenzyme A, cysteine, cocarboxylase, coenzyme I, and $MgCl_2$	0		
2X cofactors	18.4	3.2	26.1

Phosphotransacetylase. With the two anaerobic extracts employed, one had no activity, whereas the activity of the other was low. Aerobic extracts were at least 3.9 times as active as anaerobic extracts, whereas adaptation of anaerobically grown cells to an oxidative metabolism resulted in the induced synthesis of this enzyme resulting in a 7-fold increase in activity (see table 9). Phosphotransacetylase activity is proportional to the amount of coenzyme A added over a very large range. The latter finding is similar to that demonstrated by Stadtman *et al.* (1951) with extracts from *C. kluyveri*.

Oxidative decarboxylation of pyruvate. The rate of oxidative decarboxylation of pyruvate by extracts of aerobically grown *P. pestis*, employing coenzyme I as a hydrogen acceptor, was approximately 6 times that of the comparable extracts from anaerobically grown cells. Aerobic adapted extracts more than regained the activity observed with aerobic extracts, indicating an adaptive synthesis of the enzyme system involved. Coenzyme A, cocarboxylase, coenzyme I, and $MgCl_2$ are essential for maximum activity (see table 2). A carbon balance performed after the reaction had proceeded for 60 minutes (during which time all the pyruvate present (20 μM) was utilized) indicated that for 2 μM of pyruvate disappearing, approximately 1 μM each of lactate, acetate, and CO_2 was formed. (Actual values per μM of pyruvate utilized were: lactate, 0.38 μM; acetate, 0.48 μM; CO_2, 0.5 μM. No ethanol was found. Products of endogenous respiration were subtracted.) The coenzyme A requirement indicates that acetyl-coenzyme A is probably the initial product of pyruvate dismutation (Korkes, 1951). Free coenzyme A required for the completion of the reaction is probably liberated through the action of an acylase or phosphotransacetylase and acetyl phosphatase.

Citrate synthesized from acetate and oxalacetate and pyruvate and oxalacetate. Employing 10 μM

TABLE 3

Citrate synthesis from acetate and oxalacetate and from pyruvate and oxalacetate

The basic system consisted of tris(hydroxymethyl)aminomethane buffer of pH 7.5 (150 μM), KCl (60 μM), $MgCl_2$ (10 μM), potassium adenosine triphosphate (10 μM), coenzyme A (0.18 μM), cysteine HCl (20 μM), sodium oxalacetate (10 μM), with additions and deletions as indicated. The reaction mixture without the cell extract was prepared in the cold and incubated at 30 C for 5 minutes. The extract was then added and the incubation continued for an additional 15 minutes. An atmosphere of 95 per cent N_2 and 5 per cent CO_2 was employed.

	μM Citrate Formed per Hr per Mg N					
	Aerobic extracts*			Anaerobic extracts*		
	no. 24	no. 26	no. 40	no. 25	no. 27	no. 42
Plus 10 μM acetate	3.4			3.3		
Plus 10 μM acetate; less coenzyme A	1.1			0.1		
Plus 240 μM acetate	19.5	15.2	35.5	3.4	0	6.3
Plus 240 μM acetate; less coenzyme A	5.1			2.0		
Plus 240 μM acetate; 2X cofactors and oxalacetate			40.0			7.8
Plus 10 μM pyruvate	0.2					
Plus 10 μM pyruvate; plus 10 μM coenzyme I	18.6	17.8		2.0	2.9	
Plus 10 μM pyruvate; plus 10 μM coenzyme I; less coenzyme A	2.3			0.6		

* On the basis of conditions of preparation, age, and storage history, aerobic extract no. 24 is comparable to anaerobic extract no. 25; no. 26 is comparable to no. 27; and no. 40 is comparable to no. 42.

of acetate and 10 μM of oxalacetate, the activity of aerobic and anaerobic extracts was low but approximately the same (table 3). However, increasing the concentration of acetate to 240 μM resulted in an approximate 6-fold increase in activity of the aerobic extract and had no effect on the activity of the anaerobic extract. Omitting coenzyme A from the system resulted in decreased activity. Doubling the concentration of cofactors and oxalacetate causes a slight increase in rate of citrate synthesis with both aerobic and anaerobic extracts. The differences between the activity of the extracts, however, were not significantly changed by this procedure. This increase in activity appears to be due to the increased concentration of KCl, cysteine, and coenzyme A.

The dependence of citrate synthesis on high acetate concentration in the aerobic extracts indicates that citrate synthesis from acetate in this extract occurs mainly, if not entirely, by way of the acetokinase reaction, and that coenzyme A is probably required for phosphotransacetylase activity, converting acetyl phosphate to acetyl-coenzyme A.

With the substitution of pyruvate for acetate, the addition of coenzyme I was required for maximum activity. Coenzyme A was also required for this reaction (table 3). The activity of the aerobic extracts was about 6 to 9 times that of the anaerobic extracts under the conditions of this test. Comparison of activity in the presence of coenzyme A, cocarboxylase, coenzyme I, inorganic phosphate, $MgCl_2$, and KCl showed similar differences between aerobic and anaerobic extracts (table 4). Doubling the concentration of cofactors and substrate increased the rate of citrate synthesis in both types of cell extracts, while further increases were slightly inhibitory (table 4).

The condensing enzyme. The activity of the condensing enzyme was measured with acetyl phosphate and oxalacetate as substrate in the presence of excess phosphotransacetylase from *C. kluyveri*, coenzyme A, cysteine, and $MgCl_2$.

TABLE 4

Citrate synthesis from pyruvate and oxalacetate

The basic system consisted of tris(hydroxymethyl)aminomethane buffer of pH 7.5 (150 μM), KCl (60 μM), $MgCl_2$ (10 μM), KH_2PO_4-K_2HPO_4 at pH 7.5 (60 μM), coenzyme A (0.18 μM), coenzyme I (10 μM), cocarboxylase (1.0 μM), cysteine (20 μM), potassium oxalacetate (10 μM), and sodium pyruvate (10 μM). Conditions were as described in table 3.

	μM Citrate Formed per Hr per Mg N	
	Aerobic extract no. 40	Anaerobic extract no. 42
Complete system	27.5	4.3
2X cofactors and substrate	35.1	7.1
3X cofactors and substrate	24.1	6.8

TABLE 5

The condensing enzyme

The complete system consisted of tris(hydroxymethyl)aminomethane buffer of pH 7.5 (150 μM), $MgCl_2$ (10 μM), coenzyme A (0.18 μM), cysteine (20 μM), lithium acetyl phosphate (10 μM), phosphotransacetylase (0.1 ml), potassium oxalacetate (10 μM), KCl (60 μM), and cell extract in a total volume of 2 ml. Conditions were as described in table 3.

	μM Citrate Formed per Hr per Mg N		
	Aerobic extract no. 40	Anaerobic extract no. 42	Aerobic adapted extract no. 43
Complete system	53.3	4.2	58.6
2X all cofactors and substrates	82.3	7.9	90.3
2X all cofactors and substrates less coenzyme A	4.9		
2X all cofactors and substrates less cysteine	62.9		
2X all cofactors and substrates less phosphotransacetylase	78.8		
2X all cofactors and substrates less phosphotransacetylase and coenzyme A	2.5		
2X all cofactors and substrates less KCl	79.6		
2X all cofactors and substrate less oxalacetate	1.1		
2X all cofactors and substrate less lithium acetyl phosphate	0.6		
3X all cofactors and substrates	80.5	7.9	

Results shown in table 5 demonstrate that aerobic extracts have approximately 13 times greater activity than corresponding anaerobic extracts. Adaptation of anaerobically grown cells to an oxidative metabolism results in an approximately 14-fold increase in enzyme activity. Doubling the concentration of cofactors and substrates had an appreciable effect on the rate of citrate synthesis with both aerobic and anaerobic extracts, whereas tripling the concentration of either caused no further increase in activity. The differences between the aerobic and anaerobic extracts, in any case, were not changed significantly. The omission of coenzyme A resulted in a large decrease in activity, demonstrating a coenzyme A requirement for this reaction. There was a large decrease in activity with the omission of cysteine although the omission of KCl had little effect. In other experiments, however, it was noted that the omission of KCl resulted sometimes in large decreases in activity. The small response to additional phosphotransacetylase indicates that the condensing enzyme itself is almost rate limiting.

Oxidative decarboxylation of α-ketoglutarate. The work of Sanadi and Littlefield (1951) indicates that the oxidative decarboxylation of α-ketoglutarate by extracts of pigeon breast muscle is dependent upon the presence of coenzyme I and coenzyme A and results in the formation of succinyl-coenzyme A, CO_2, and reduced coenzyme I. *P. pestis* appears to possess a similar α-ketoglutarate decarboxylase system. Maximum rate of activity was achieved only with the addition of all of the above cofactors (table 6). Extracts from aerobic cells were 4 to 10 times as active as comparable extracts from anaerobic cells, and adaptation of anaerobically grown cells to an oxidative metabolism resulted in significant increases in activity.

TABLE 6

Oxidative decarboxylation of α-ketoglutarate

The reaction mixture contained potassium phosphate buffer of pH 7.4 (66 μM), α-ketoglutarate (20 μM), $MgCl_2$ (10 μM), coenzyme I (10 μM), coenzyme A (0.18 μM), cysteine HCl (20 μM), cocarboxylase (1 μM), and cell extract to a total volume of 2.7 ml. The reaction was carried out under N_2 at 30 C. The rate of α-ketoglutarate dehydrogenase is based upon the amount of CO_2 evolved during the first 5 minutes after addition of α-ketoglutarate.

	μM α-Ketoglutarate Decomposed per Hr per Mg N					
	Aerobic extracts*		Anaerobic extracts*			Aerobic adapted extract no. 28*
	no. 33	no. 26	no. 35	no. 27	no. 42	
Complete system	3.00	4.91	0.31	1.18	1.07	4.50
Less $MgCl_2$	0.46					
Less coenzyme I	1.37					
Less coenzyme A, cysteine	2.49					
2X cofactors	3.17				0.63	

* On the basis of conditions of preparation, age, and storage history, aerobic extract no. 33 is comparable to anaerobic extract no. 35; and extracts no. 26, no. 27, and no 28 are comparable.

Succinic dehydrogenase. There appears to be no significant difference in succinic dehydrogenase activity between the different types of extracts (table 9). Coenzyme A, adenosine triphosphate, coenzymes I and II, flavin adenine dinucleotide, flavin adenine mononucleotide, inorganic phosphate, and cytochrome c failed to stimulate succinic dehydrogenase activity. Employing oxygen as the hydrogen acceptor in the absence of added coenzyme I, succinate is oxidized incompletely with the accumulation of fumarate and malate, accounting for 52 and 34 per cent, respectively, of the succinate utilized. The production of malate is probably the result of fumarase activity (Englesberg *et al.*, 1954*b*). With the addition of coenzyme I, succinate is oxidized to acetate and CO_2 (table 7).

Malic dehydrogenase. Extracts from aerobically grown cells were approximately 3 times as active as the comparable extracts of anaerobically grown cells, and the latter cells adapted to an oxidative metabolism demonstrated an approximately 3-fold increase in enzyme activity (table 9). Doubling the concentration of substrate and cofactors resulted in no increase in rate.

Tests for possible inhibitory factor(s) in the anaerobic extract. The possibility exists that both aerobic and anaerobic cell extracts contain the same quantity of oxidative enzymes and that the relatively low activity of the anaerobic extract observed is due to the presence of an enzyme inhibitor or inhibitors. To test for the presence of such inhibitor(s), the effect of the anaerobic extract on isocitric dehydrogenase activity of aerobic extracts was determined by comparing the activity of the aerobic extract alone and when mixed with an anaerobic extract. Isocitric dehydrogenase was chosen for testing since the differences in activities between aerobic and anaerobic extracts were greatest with respect to this enzyme (Englesberg *et al.*, 1954*b*), and therefore the inhibitory effect presumably would be greater and more easily demonstrable. Figure 2 shows that the anaerobic extract has no inhibitory effect on the isocitric dehydrogenase activity of the aerobic extract. In another experiment it has been shown that the rate of citrate oxidation (oxygen as the final hydrogen acceptor) by an aerobic extract, which was approximately 60 times as rapid as that of a comparable anaerobic extract, was not decreased by the addition of

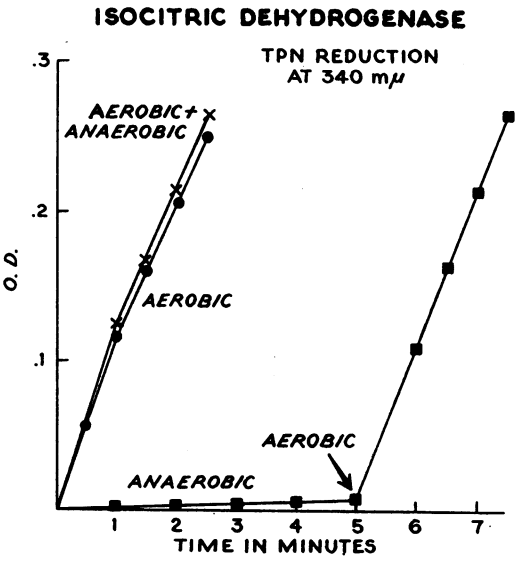

Figure 2. A test for the presence of an inhibitory substance in the anaerobic cell-free extracts. The isocitric dehydrogenase activities of the aerobic extract, anaerobic extract, of a mixture of both extracts, and of the aerobic extract added 5 minutes after the addition of the anaerobic extract were measured by the reduction of triphosphopyridine nucleotide at 340 mμ (Englesberg *et al.*, 1954*b*).

equal amounts of anaerobic extract. Since citrate is oxidized to acetate under the conditions of this experiment, it appears that the anaerobic extract does not possess inhibitors against the several enzymes involved in this conversion.

Specificity of the adaptive phenomenon—glyceraldehyde phosphate dehydrogenase, phosphofructokinase. The induced synthesis of phosphotransacetylase, pyruvate decarboxylase system, condensing enzyme, isocitric dehydrogenase,[5] aconitase,[5] α-ketoglutarate decarboxylase system, fumarase,[5] and malate dehydrogenase may be taken as indicative of the utilization of these enzymes in the complete oxidation of glucose and in the oxidation of acetate if it can be shown that they are specifically synthesized as a result of the adaptation and are not just the result of an all-over increase in enzymatic activity due to aeration. To determine whether this increase in enzymatic activity is specific, assays were made for glyceraldehyde phosphate dehydrogenase and phosphofructokinase in the aerobic, an-

[5] See Englesberg *et al.* (1954*b*).

TABLE 7

Incomplete oxidation of pyruvate and some tricarboxylic acid cycle compounds by aerobic cell extracts

The complete system contained substrate (10 μM), phosphate buffer, pH 7.0 (66 μM), coenzyme I (2 μM), adenosine triphosphate (2 μM), $MgSO_4$ (2 μM), and cell extract in a total volume of 2.6 ml. KOH was employed in the center well in vessels used for measuring O_2 uptake, while 4 N H_2SO_4 was employed in the side arm for measuring CO_2 production. The Warburg experiment was carried out at 30 C. Analytical determinations were performed on the supernatant of vessels used in determining biologically produced CO_2. Endogenous CO_2, acetic and lactic acid production, and O_2 uptake were estimated in each case and subtracted.

Substrate	μM Products per μM Substrate Utilized			
	O_2 uptake	CO_2 evolution	Acetate	Lactate
Pyruvate*	0.20	0.65	0.70	0.28
Oxalacetate*	0.48	1.76	0.82	0.14
Malate	0.86	2.01	0.80	0
Succinate	1.27	1.66	0.74	0
α-Ketoglutarate	1.81	2.76	0.70	0
Citrate	2.35	3.56	0.80	0

* In experiments with pyruvate and oxalacetate as substrate, values are those obtained at the first significant decrease in rate of O_2 uptake, while for the remaining substrates values are those obtained when O_2 uptake was almost zero after the subtraction of endogenous respiration. This probably accounts for the accumulation of lactate from both pyruvate and oxalacetate.

aerobic, and aerobic adapted extracts previously described. The results of these assays are shown in table 9. The anaerobic extract has approximately 2.4 times as much glyceraldehyde phosphate dehydrogenase activity as the corresponding aerobic extract, and adaptation to aerobic metabolism results in a decrease of 1.9 times in activity. Similarly, with regard to phosphofructokinase, anaerobic extracts are 2 times as active as the corresponding aerobic extracts, and adaptation to aerobic metabolism results in a decrease of 1.6 times in activity. The specificity of the adaptation was also evident by the similarity in rates of acetate activation and oxalacetate decarboxylation by all three types of cell extracts (table 9).

The failure of cell extracts to oxidize acetate. Although the aerobic extracts possess all the enzymes required for the oxidation of acetate and aerobically grown resting cells oxidize acetate rapidly, the cell extracts failed to oxidize this substrate. This inability of the extracts to oxidize acetate is reflected by the incomplete oxidation of glucose, pyruvate, citrate, α-ketoglutarate, succinate, fumarate, malate, and oxalacetate (Englesberg *et al.*, 1954*b*). Further analysis for products of oxidation of several of these substrates has demonstrated the accumulation of approximately 0.8 μM of acetate per μM of substrate oxidized (table 7). The possibility that the inability to oxidize acetate and the incomplete oxidation of the compounds listed above are partially the result of the destruction of oxalacetate, thereby inhibiting or preventing the condensation reaction, is indicated by the presence of an active oxalacetate decarboxylase in the cell extracts (table 9). Another probable factor causing this failure to oxidize acetate is the requirement for large concentrations of acetate to effect its activation (see figure 1). The possibility existed, therefore, that by loading the system with large quantities of acetate it

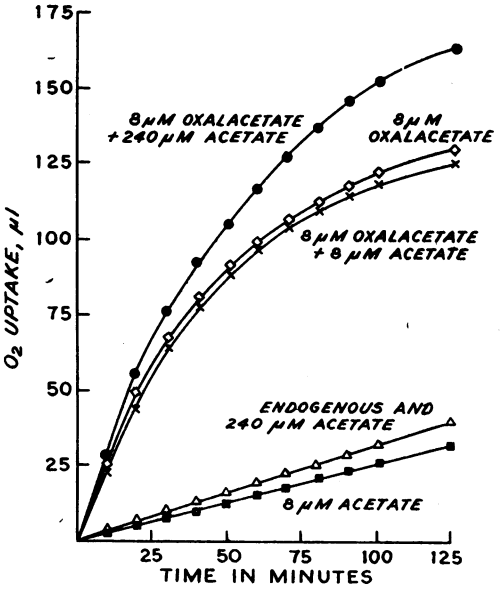

Figure 3. The stimulatory effect of high concentrations of acetate on oxalacetate oxidation. The complete system contained cell-free extract (2 ml), phosphate buffer, pH 7 (66 μM), coenzyme I (2 μM), adenosine triphosphate (2 μM), $MgSO_4$ (2 μM), and substrates as indicated.

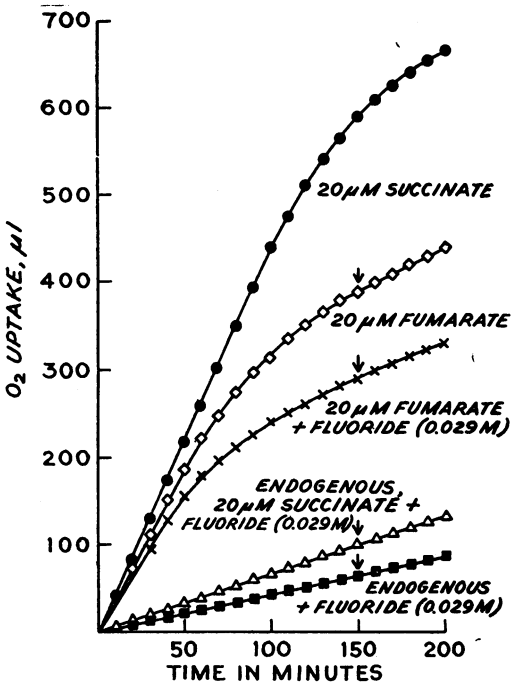

Figure 4. The oxidative production of succinate from fumarate employing potassium fluoride as an inhibitor of succinate oxidation. The arrows indicate the point at which the reaction was stopped for determination of products of the reaction.

should be possible to push the synthesis of acetyl phosphate and demonstrate a stimulation of oxalacetate oxidation. As demonstrated in figure 3, there is no oxidation of acetate when present alone in either 8 or 240 µM quantities. The oxygen uptake with 8 µM of oxalacetate is the same with or without the addition of 8 µM of acetate. However, the addition of 240 µM of acetate resulted in a marked stimulation of oxalacetate oxidation. The total oxygen uptake, however, is still far below that required for the complete oxidation of oxalacetate, probably as the result of the destruction of oxalacetate by the decarboxylase.

Fluoride inhibition of succinate oxidation and the oxidative production of succinate from fumarate. Fluoride has been shown to be a potent inhibitor of succinate dissimilation (Englesberg *et al.*, 1954*b*) at concentrations that have no significant effect on the initial oxidation of other tricarboxylic acid cycle compounds. If cycling is occurring in the oxidation of fumarate, for instance, it should be possible to demonstrate a partial inhibition of oxygen utilization and the accumulation of succinate by employing fluoride as an inhibitor. Figure 4 demonstrates that succinate oxidation is almost completely inhibited by 0.029 M of potassium fluoride. Fluoride also inhibits the endogenous respiration about 35 per cent and fumarate oxidation by 22.5 per cent, as estimated 150 minutes after the initiation of the reaction (the respective endogenous oxygen uptake has been subtracted in determining the latter value). Analysis for products of fumarate oxidation at 150 minutes (see figure 4 and table 8) demonstrates a sizable accumulation of succinate by fluoride inhibited extracts with a corresponding decrease in amount of acetate and CO_2 produced.

TABLE 8

The oxidative production of succinate from fumarate employing potassium fluoride as an inhibitor of succinate oxidation

Products of Fumarate Oxidation	µM Products per µM Fumarate Utilized	
	Fumarate	Fumarate + fluoride
CO_2	1.59	1.48
Acetate	0.79	0.29
Lactate	0.23	0.26
Succinate	0	0.18
Oxygen utilized	0.65	0.53
Carbon recovery (%)	98.5	89.4
Redox index	0.97	1.03

DISCUSSION

Resting cell suspensions of *P. pestis*, grown aerobically in a casein hydrolyzate mineral glucose medium, oxidize pyruvate, acetate, and the C4 dicarboxylic acids at fairly rapid rates, and glucose is oxidized rapidly and "completely". In contrast, anaerobically grown cells are unable to oxidize acetate and oxidize glucose slowly and incompletely with the accumulation of pyruvate and other fermentation end products. This incomplete oxidation of glucose and failure to oxidize acetate are accompanied by large decreases in rates of oxidation of pyruvate and the C4 dicarboxylic acids. Aeration of anaerobically grown cells in a glucose casein hydrolyzate mineral medium results in the conversion of these cells to the physiological behavior of aerobically grown cells under conditions which eliminate mutation and selection as

TABLE 9

Comparison of the enzymatic activities of aerobic, anaerobic, and aerobic adapted extracts of Pasteurella pestis strain A1122

(Summary)

	A Aerobic	B Anaerobic	$\frac{A}{B}$	C Aerobic Adapted	$\frac{C}{B}$
1. Oxidative decarboxylation of pyruvate	21.9	3.7	5.9	25.4	6.9
2. Phosphotransacetylase	23.5	6.1	3.9	42.4	7.0
3. Condensing enzyme	82.3	7.9	10.4	90.3	11.4
4. Isocitric dehydrogenase*	73.6	1.2	61.2		
		3.5		66.4	18.9
5. Aconitase*	2.4	0.3	8.0		
		0.1		1.2	12.0
6. Oxidative decarboxylation of α-keto-	3.0	0.3	10.0		
glutarate	4.9	1.2	4.1	4.5	3.8
7. Fumarase*	50.3	11.1	4.5		
		4.3		46.9	10.9
8. Malic dehydrogenase	13.4	4.9	2.7	12.2	2.5
	14.3	3.9	3.7	16.1	4.1
9. Cytochrome (560 mμ)*	+++	−	+++	+++	+++
10. Succinic dehydrogenase	1,030	555	1.9		
		1,540		1,460	0.9
11. Acetokinase	23.4	16.3	1.4		
	25.2	30.3	0.8	32.5	0.9
	17.5	20.0	0.9	19.8	1.0
12. Oxalacetate decarboxylase	6.9	4.5	1.5	4.6	1.0
13. Glyceraldehyde phosphate dehydro-	48.1	113.6	0.4	61.4	0.5
genase					
14. Phosphofructokinase	22.9	45.6	0.5	28.1	0.6

Activity is represented as the number of μM of substrate transformed or product synthesized per hr per mg N.

* See Englesberg *et al.* (1954*b*).

being the cause of the adaptive response (Englesberg *et al.*, 1954*a*). Enzymatic analysis indicates that the change from anaerobiosis to aerobiosis results in the specific induced synthesis of isocitric dehydrogenase, aconitase, fumarase, cytochrome (Englesberg *et al.*, 1954*b*), phosphotransacetylase, the condensing enzyme, malic dehydrogenase, and of enzymes involved in the oxidative decarboxylation of pyruvate and α-ketoglutarate (see table 9 for summary).

That this adaptive response is not the result of the production of inhibitor(s) by anaerobically grown cells and the loss of the inhibitor(s) by aerobic adaptation was shown by the failure of extracts of anaerobically grown cells to inhibit the isocitric dehydrogenase activity as well as other enzymes of the aerobic extract involved in the oxidation of citrate to acetate.

The specificity of the adaptive phenomenon was indicated by the findings that adaptation to this aerobic metabolism resulted in decreases in phosphofructokinase and glyceraldehyde phosphate dehydrogenase and that the acetokinase and oxalacetate decarboxylase activity remained essentially the same (table 9).

This specific induced biosynthesis of enzymes 1 to 9 as described in table 9 and mentioned above, correlated with the change from inability to oxidize acetate, incomplete oxidation of glucose, and slow rates of oxidation of pyruvate and the C4 dicarboxylic acids to the rapid oxidation of acetate, pyruvate, and the C4 dicarboxylic acid compounds and the rapid and "complete" oxidation of glucose, implicates these enzymes and the tricarboxylic acid cycle as required for acetate and "complete" glucose oxidation. Although there is no adaptive synthesis of succinic dehydrogenase, other evidence indicates that it

is involved: (1) the succinic dehydrogenase activity of the cell extracts is extremely high; (2) fluoride, which is a potent inhibitor of succinic oxidation by *P. pestis*, produces a partial inhibition of citrate and α-ketoglutarate oxidation with the resulting accumulation of succinate (Englesberg *et al.*, 1954*b*); (3) the omission of coenzyme I, which is required for malate oxidation, results in the incomplete oxidation of succinate with the accumulation of fumarate and malate; and (4) the oxidation of fumarate in the presence of fluoride leads to the oxidative production of succinate.

The oxidative production of succinate from fumarate in the presence of fluoride confirms the cycling phenomenon and adds further support to the functioning of the tricarboxylic acid cycle in aerobic *P. pestis*. Other evidence was also presented supporting the operation of this cycle: (1) the cofactor requirement of each of the enzymes studied, as well as the products identified, is the same as those shown to be involved in the tricarboxylic acid cycle; (2) citrate is synthesized from acetate and oxalacetate and from pyruvate and oxalacetate at rates comparable to that for the synthesis of citrate from active acetate and oxalacetate. Although it has been shown that anaerobically grown *Saccharomyces cerevisiae* differ significantly in the levels of activity of various oxidative enzymes from cells grown aerobically, the functioning of the tricarboxylic acid cycle and its adaptive control has not as yet been established in this organism (Hirsch, 1952).

The high phosphotransacetylase activity of aerobically grown cells utilizing glucose as a carbon source indicates that free acetate may be a "normal" product of glucose oxidation. This is also borne out by the relatively high acetokinase activity.

Both aerobically and anaerobically grown *P. pestis* strain A1122 possess a highly active glyceraldehyde phosphate dehydrogenase and phosphofructokinase, suggesting that the Embden-Meyerhof pathway is operative in both the aerobic and anaerobic metabolism of glucose. That this is the only pathway operating in the glucose metabolism of anaerobically grown cells is doubtful, for it has previously been shown that anaerobically grown resting cells, given glucose in the presence of oxygen, oxidize glucose incompletely, producing CO_2 at a rapid rate with the simultaneous production of pyruvate. The rate of CO_2 produced from glucose finally drops to the slow rate characteristic of pyruvate dissimilation by this organism (Englesberg *et al.*, 1954*a*).

The induced biosynthesis of the series of fundamentally important enzymes described above indicates the marked control of environmental factors over the enzyme forming mechanisms, and serves as another example of sequential enzymatic induction (Stanier, 1951). Although the presence or absence of oxygen appears to be responsible for the enzymatic changes indicated here, the mechanisms require considerable elucidation. Oxygen may play the role of substrate inducer of the terminal cytochrome, as explained by Slonimski (1953), and this cytochrome might in turn serve as the substrate inducer of the next enzyme in the chain of electron transport. However, it is difficult to explain on this basis the effect of oxygen on enzymes such as phosphotransacetylase, condensing enzyme, fumarase, isocitric dehydrogenase, and on other enzymes in the tricarboxylic acid cycle. Both aerobic and anaerobic cell extracts of *P. pestis* strain A1122 possess similar amounts of acetokinase and have similar activity with regard to the noncoenzyme A acetyl phosphate decomposition; yet the aerobic cell extract possesses about four times as much phosphotransacetylase. Since anaerobic cells produce acetate as a product of glucose fermentation, sufficient acetyl phosphate would seem to be available for the synthesis of phosphotransacetylase; yet the amount of this enzyme present is below that of the aerobic cell. One might look at the fumarase activity in a similar light. Both cells produce succinate, and both have equal succinic dehydrogenase activity; yet the aerobic cell extract has 5 to 10 times as much fumarase. Although this does not provide any proof that the particular intermediates were present and in sufficient concentration, however small, for induced enzyme synthesis, it is apparent that the presence of oxygen may be required for the inducing system to respond to the particular substrates or that the absence of oxygen inhibits the accumulation of a sufficient quantity of these inducers.

SUMMARY AND CONCLUSIONS

Anaerobically grown *Pasteurella pestis* strain A1122, when adapted to an oxidative metabolism (i.e., to the oxidation of acetate, rapid oxidation

of pyruvate and the C4 dicarboxylic acids, and the rapid and "complete" oxidation of glucose), demonstrate a specific induced synthesis of phosphotransacetylase, enzyme system for the oxidative decarboxylation of pyruvate, condensing enzyme, isocitric dehydrogenase, aconitase, enzyme system for the oxidative decarboxylation of α-ketoglutarate, fumarase, malic dehydrogenase, and cytochrome, thereby implicating these enzymes and the tricarboxylic acid cycle as required for acetate oxidation and for the "complete" oxidation of glucose.

The oxidative production of succinate from fumarate employing fluoride as an inhibitor of succinate oxidation, the high succinic dehydrogenase activity, the isolation of malate and fumarate as products of succinate oxidation, together with the isolation of products of other enzyme reactions and the identification of the cofactor requirements for each reaction, further support this conclusion.

The specificity of the adaptation is indicated by the corresponding decrease in activity of phosphofructokinase and glyceraldehyde-phosphate dehydrogenase as a result of this aerobic adaptation and by the similarity in acetokinase and oxalacetate decarboxylase activity in both aerobic and anaerobic cell-free extracts.

Cell-free extracts of both aerobically and anaerobically grown cells activate acetate by an adenosine triphosphate, magnesium dependent acetokinase reaction. The failure of the aerobic extract to oxidize acetate, although it possesses all the enzymes to do so, and the incomplete oxidation of the tricarboxylic acid compounds with the accumulation of acetate appear to be the result of the destruction of oxalacetate by an oxalacetate decarboxylase which inhibits or prevents the condensation reaction, the liberation of free acetate, and the large concentration of acetate required to effect a significant activation.

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