Oxalacetate Decarboxylase of *Aerobacter aerogenes*. I. Inhibition by Avidin and Requirement for Sodium Ion*

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ABSTRACT: The oxalacetate (OAA) decarboxylase (OAA 4-carboxy-lyase) present in extracts of *Aerobacter aerogenes* did not require added divalent cation for its activity and was not inhibited by 0.1 M ethylenediaminetetracetate (EDTA). The decarboxylase was inhibited 95% by avidin (0.5 unit), but not by avidin pretreated with biotin, indicating that it is a biotinoprotein. After dialysis or salt fractionation, the enzyme was largely (90%) inactivated and could be reactivated by the addition of Na^+. K^+, NH_4^+, Li^+, Rb^+, and Cs^+ could not replace Na^+. The K_m for OAA was about 10^{-3} M. The decarboxylase was inhibited by cyanide and 8-hydroxyquinoline but not by ouabain. This avidin-sensitive, Na^+-requiring OAA decarboxylase was specifically induced by growth on citrate, either anaerobic or aerobic, since the OAA decarboxylase activity of *A. aerogenes* cells grown on glucose or glycerol was very low and not sensitive to avidin, EDTA, or Na^+.

The anaerobic dissimilation of citric acid by cell suspensions of *Aerobacter indologenes* (Brewer and Werkman, 1939) and by cell-free extracts of *Aerobacter aerogenes* (Dagley and Dawes, 1935a) involves the cleavage of citrate to oxalacetate (OAA)[1] and acetate followed by the decarboxylation of OAA to pyruvate. This paper demonstrates that the OAA decarboxylase (OAA 4-carboxy-lyase) of *A. aerogenes* is a novel inducible enzyme of this type which is sensitive to avidin but not to EDTA. Moreover, it specifically requires sodium ion for activity.

Experimental Section

Cell Growth and Extraction. A. aerogenes NCTC 418 was grown without aeration at 37° in 20-l. bottles filled to the neck with medium above which a 1-in. layer of sterile mineral oil was placed. The medium used contained per liter of distilled water: monobasic potassium phosphate, 2 g; ammonium sulfate, 1 g; magnesium sulfate·$7H_2O$, 0.4 g; and 21 g of trisodium citrate·$2H_2O$ (Merck, reagent grade). It was adjusted to pH 7.0 with 7 N sodium hydroxide. A 1% inoculum of similarly grown cells was added and after 24 hr the newly grown cells were cooled to stop gas production and harvested with a refrigerated Sharples centrifuge. The harvested unwashed cells were stored at −20° for up to 8 months before extraction without significant loss of enzyme activity. Under these conditions of growth about 18 g of citrate/l. was decomposed in 24 hr.

In some experiments glucose or glycerol (1%) was substituted for citrate as sole carbon source. These cells, grown in 9 mM Na^+, were washed twice after harvesting. In aerobic growth experiments, the 20-l. bottles were aerated vigorously by passing filtered air under pressure through a sintered-glass disk placed near the bottom of the bottle. The pH values of the medium at time of harvest were 6.4 (citrate, anaerobic), 8.5 (citrate, aerobic), 5.5 (glycerol), and 4.5 (glucose).

Enzyme extracts were prepared by suspending frozen cells in three volumes (per gram wet weight of cells) of 0.02 M PO_4(K) buffer (pH 7.0) containing 5 mM $MgCl_2$ and 5 mM glutathione and sonicating the suspension for 5 min in a Branson sonifier at 2–8°. The suspension was spun for 45 min at 26,000*g*. The clear yellow supernatant which contained 10–12 mg of protein/ml was used in all experiments unless otherwise stated.

Methods. Measurement of OAA decarboxylase activity was carried out in test tubes containing Tris-HCl or potassium phosphate buffer (pH 8.0) (100 μmoles), OAA, enzyme, and other components in a final volume of 1.0 ml. All components save enzyme and OAA were mixed at room temperature. Then cold enzyme was added and the reaction mixture was placed in a 30° bath. After 5 min, 0.10 ml of dipotassium OAA (~0.1 M) was added to start the reaction which was terminated after 5–10 min by addition of 0.20 ml of 30% metaphosphoric acid. After removal of the precipitate by centrifugation, the residual OAA and pyruvate in a sample of the clear supernatant was determined spectrophotometrically with DPNH and crystalline L-malate and L-lactate dehydrogenases at pH 6.5. Since the L-malate dehydrogenase was free of L-lactate dehydrogenase but the latter contained significant traces of the former, it was essential to

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[1] Abbreviations used: OAA, oxalacetate; diol buffer, 2-amino-2-methyl-1,3-propanediol–HCl buffer; TPN, oxidized triphosphopyridine nucleotide; acetyl-CoA, acetyl coenzyme A; DPNH, reduced diphosphopyridine nucleotide; ADP and ATP, adenosine di- and triphosphates.

measure residual OAA first and then pyruvate. Specific activity refers to micromoles of pyruvate formed per minute per milligram of protein under standard conditions. Unless otherwise stated, pyruvate values have been corrected by an amount equal to the sum of the zero-time pyruvate and the pyruvate formed by chemical decarboxylation during the incubation.

In some experiments the decarboxylation of OAA was measured directed by following its disappearance at 290 mμ in a Gilford automatic spectrophotometer.

Kochsaft refers to the clear supernatant solution obtained by placing the sonic extract of *A. aerogenes* in a boiling-water bath for 3 min and removing the precipitate by centrifugation for 20 min at 26,000*g*. Protein was determined by the biuret method (Gornall *et al.*, 1949).

Other Enzyme Assays. Citritase was assayed by measuring OAA and pyruvate formation from citrate at pH 8.0 with 8 mM $MgCl_2$. All other assays were spectrophotometric. Malate and lactate dehydrogenases were assayed at pH 6.5 by following DPNH oxidation by OAA and pyruvate, respectively. TPN reduction by *d*-isocitrate and L-malate, with added 2.5 mM $MgCl_2$, was measured at pH 8.0 and 8.8, respectively. Dehydration of *d*-isocitrate and L-malate was measured at pH 8.0 and 240 mμ.

Materials. All dehydrogenases were products of Boehringer and Co. Trisodium *dl*-isocitrate was purchased from Calbiochem. Oxalacetic acid (*cis*-enol) (mp 150–152°) (Sigma Chemical Co.) was dissolved in water and neutralized immediately before use. This solution contained about 2% pyruvic acid and was free of Na^+. Values of added OAA given are those determined by enzyme assay. Avidin (12 units/mg) was purchased from Worthington Biochemical Corp.

Results

Insensitivity of OAA Decarboxylase to EDTA. The capacity of extracts of citrate-grown *A. aerogenes* to decarboxylate OAA to pyruvate was first noted by Dagley and Dawes (1953a). As shown in Table I, 10 mM EDTA did not inhibit OAA decarboxylase activity of the extract, nor did 4 mM Mg^{2+} affect decarboxylase activity. It will be noted that the sum of pyruvate and OAA recovered equalled the OAA added, *i.e.*, no further metabolism of pyruvate occurred under the experimental conditions. In other experiments, even 100 mM EDTA caused no inhibition of enzyme activity.

Extracts of *A. aerogenes* contained L-malic–TPN enzyme but the activity of this enzyme was completely inhibited by 3 mM EDTA. Thus, L-malic enzyme did not contribute to the OAA decarboxylase activity of the extract.

Sensitivity of OAA Decarboxylase to Avidin. OAA decarboxylase was almost completely inhibited by small amounts (0.5 unit) of avidin (Table II). This inhibition was evident both when OAA was added as such or generated from citrate by endogenous citritase. In the absence of avidin only pyruvate accumulates from the citrate cleavage reaction since decarboxylase activity of the extract is in excess of citritase activity. With addition of avidin, OAA, which is the primary product (with acetate) of citrate cleavage, accumulated. After pretreatment of the avidin solution with biotin, no inhibition of decarboxylase was evident, showing that the inhibition was due to avidin and not to impurities. The sensitivity of the decarboxylase to avidin was

TABLE I: Failure of EDTA to Inhibit Oxalacetate Decarboxylase.

Additions[a]	Oxalacetate (μmoles)	Pyruvate (μmoles)
OAA	4.26	4.24
OAA + EDTA	4.36	4.43
OAA + Mg^{2+}	4.65	4.17

[a] The reaction mixture contained: $PO_4(K)$ buffer (pH 8.0, 100 μmoles), potassium OAA (8.75 μmoles), *A. aerogenes* extract (1.25 mg), and, where indicated, Tris-EDTA, (10 μmoles) or $MgCl_2$ (4 μmoles). The volume was 1.0 ml. The incubation was for 5 min at 30°.

TABLE II: Inhibition of Oxalacetate Decarboxylase by Avidin.

Additions[a]	Oxalacetate (μmoles)	Pyruvate (μmoles)	Inhibn (%)
	Expt 1		
Citrate	0	1.69	
Citrate + avidin	1.25	0.29	83
OAA	4.26	5.66	
OAA + avidin	8.25	0.65	92[b]
OAA (no enzyme)	8.57	0.23	
	Expt 2		
OAA	4.03	5.29	
OAA + avidin	8.64	0.65	90[b]
OAA + avidin + biotin	4.42	5.07	4[b]

[a] The reaction mixture contained (expt 1): $PO_4(K)$ buffer, pH 8.0, 100 μmoles; *A. aerogenes* extract, 2.5 mg with citrate, 1.25 mg with OAA; and as indicated, potassium citrate, 10 μmoles; potassium OAA, 8.75 μmoles; and avidin, 0.5 unit. The volume was 1.0 ml. In expt 2, 9.3 μmoles of potassium OAA was added and 0.2 mg of biotin was added to reaction mixture 5 min before enzyme addition. The incubation was for 5 min at 30°. [b] Corrected for nonenzymatic decarboxylation.

TABLE III: Inactivation of Oxalacetate Decarboxylase by Dialysis and Restoration of Activity by Kochsaft.

Age (hr)	Enzyme Fraction[a] Treatment	Sp Act. Alone	Sp Act. +Kochsaft	Stimulation by Kochsaft (%)
2	Initial extract	0.68		
24	30–50 ammonium sulfate, undialyzed	0.37		
24	30–50 ammonium sulfate, dialyzed 1.5 hr	0.24	0.67	280
48	30–50 ammonium sulfate, dialyzed 1.5 hr	0.27	0.68	250
120	30–50 ammonium sulfate, dialyzed 1.5 hr	0.12	0.31	260
120	30–50 ammonium sulfate, redialyzed 6 hr	0	0.32	

[a] The assay mixture contained: KPO_4 buffer, pH 8.0, 100 μmoles; potassium OAA, 8.75 μmoles; enzyme, and where indicated 0.1 ml of *A. Aerogenes* kochsaft. The volume was 1.0 ml. The incubation was for 5 min at 30°.

great; even 0.006 unit of avidin caused 12% inhibition of activity.

Sodium Requirement of Decarboxylase. When the sonic extract was treated with ammonium sulfate, and the fraction precipitating between 30 and 50% saturation was redissolved in buffer, the specific activity of the decarboxylase decreased by almost 50% (Table III). Brief dialysis of this salt fraction caused a further decrease in activity. The activity of the dialyzed salt fraction could be restored to that of the original extract by addition of a kochsaft prepared from the original extract. After 4-days' frozen storage, the dialyzed salt fraction lost about half its endogenous activity, but kochsaft addition gave the same proportionate increase in activity as before, showing that the loss of activity on storage was due to partial enzyme inactivation and not due to loss of sensitivity of this enzyme preparation to the activator present in the kochsaft. Further dialysis of the aged fraction gave a preparation which was inactive in the absence of kochsaft.

Preliminary experiments showed that the partly inactivated decarboxylase retained its avidin sensitivity both before and after activation by kochsaft. Moreover, kochsaft could not be replaced by a variety of coenzymes including biotin, cocarboxylase, CoA, and acetyl-CoA, by the standard amino acids, potassium acetate or citrate, or adenine nucleotides. The activity of kochsaft was not affected by charcoal treatment. Treatment of the kochsaft with various resins showed that the cofactor activity of the kochsaft was adsorbed by Dowex 50 (H^+) form, but not by Dowex 1 (Cl^-) or by DEAE-cellulose. Thus, the cofactor was a cation. However, the divalent cations Mg, Mn, Co, Zn, and Fe were inactive. Further insight into the probable nature of the cofactor resulted from the observations that both the growth medium itself and the spent medium after complete dissimilation of the citrate originally present could replace kochsaft as cofactor for the decarboxylase. This implicated a monovalent cation, Na^+ or NH_4^+. Various monovalent cations were therefore tested as activators and, as shown in Table IV, Na^+ was found to specifically activate OAA decarboxylase activity. Repeated experiments demonstrated that Li^+, NH_4^+, Rb^+, Cs^+, and K^+ (tested with Tris–OAA as substrate) were inactive at low or high concentrations. When tested at 0.01 M concentration, K^+ and particularly Li^+ caused significant inhibition of enzyme activation by 0.02 M Na^+ (Table V); NH_4^+, Rb^+, and Cs^+ were noninhibitory under these conditions.

Effect of Na^+ Concentration. With one exception (*cf.* Table II), after treatment of the extract by salt fractionation and dialysis or by passage over Dowex 50 (H^+), or Sephadex G-50, there always remained some residual decarboxylase activity (about 15–20% of maximal activated rate) in the absence of added Na^+, whether phosphate or Tris buffers were employed in dissolving the enzyme or in the reaction mixture. While contaminating Na^+ in these materials (estimated to contribute 0.5–1.0 μmole of Na^+/ml of reaction mixture) could account for this residual activity, it is not ruled out

TABLE IV: Effect of Monovalent Cations on Decarboxylation of Oxalacetate to Pyruvate.

Cation[a]	Pyruvate (μmoles) 0.5 mM[b]	8 mM[b]
None	0.76	0.73
Sodium	1.84	4.96
Lithium	0.78	0.69
Ammonium	0.76	0.98
Rubidium	0.72	0.98
Cesium	1.26	0.90

[a] The reaction mixture contained: KPO_4 buffer, pH 8.0, 100 μmoles; 9.5 μmole of potassium OAA; *A. aerogenes* 30–50 ammonium sulfate fraction, 1.4 mg; and cation (chloride salt) as indicated. The volume was 1.0 ml. The incubation was for 10 min at 30°. [b] Concentration of cation.

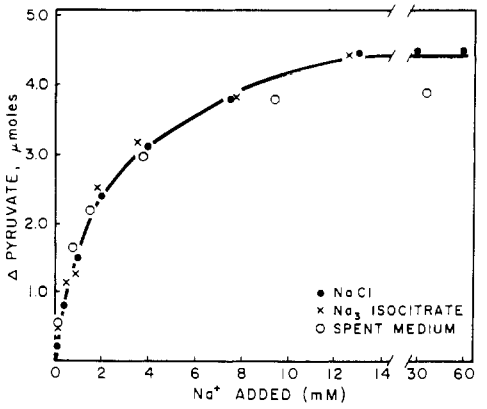

FIGURE 1: Effect of Na^+ concentration on OAA decarboxylase activity. Pyruvate formation has been corrected for the amount formed (0.71 μmole) in absence of added Na^+. Standard assay conditions, potassium phosphate buffer. Enzyme was dialyzed 30–50 ammonium sulfate fraction (1.2 mg). Na^+ of spent medium was determined by flame photometry.

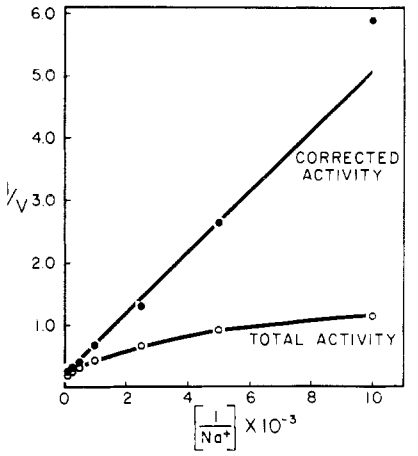

FIGURE 2: Lineweaver–Burk plot of kinetic data in Figure 1.

that traces of Na^+ may remain tightly bound to the enzyme.

The effect of added Na^+ concentration on OAA decarboxylase activity is shown in Figure 1. In this figure the cation was added (1) as NaCl, (2) as trisodium *dl*-isocitrate, or (3) as citrate-free spent medium. It is seen that in each case the increment in activity (above the residual activity) with increasing Na^+ concentration was identical, demonstrating that the activity of the spent medium is accounted for completely by its Na^+ content and that Na^+ activation is independent of the anion added with it. As little as 0.1 mM added Na^+ produced measurable activation of the decarboxylase and 12 mM added Na^+ gave maximum activation.

Figure 2 shows a Lineweaver–Burk analysis of the data in Figure 1. On plotting the reciprocal of the *observed* rate of decarboxylase activity against the reciprocal of the Na^+ concentration, the lower curve was obtained. When observed rates were corrected for the residual rate in absence of added Na^+, then a straight line was obtained. The calculated K_m for *added* Na^+ was 10^{-3} M.

Substrate Affinity. The rate of decarboxylation of OAA increased with increasing concentrations of OAA up to about 7 mM OAA (Figure 3). The apparent Michaelis–Menten constant (K_m) for OAA calculated from a Lineweaver–Burk plot (Figure 3, insert) was 3.73×10^{-4} M. The OAA decarboxylases of *Micrococcus lysodeitkicus* (Herbert, 1951) and *Acetobacter xylinum* (Benziman and Heller, 1964) have K_m values of 2 and 2.8×10^{-3} M, respectively. The OAA decarboxylase activity of *Lactobacillus arabinosus* and of wheat germ L-malic enzymes gave K_m values of $\sim 10^{-2}$

TABLE V: Inhibition by Li^+ and K^+ of Na^+ Activation of Decarboxylase.

Cations Present[a]	Pyruvate (μmoles)
Na^+	4.15
Na^+ and K^+	3.76
Na^+ and Li^+	2.71
Na^+ and NH_4^+	4.15
Na^+ and Rb^+	3.95
Na^+ and Cs^+	4.05

[a] The reaction mixture contained: Tris-HCl buffer, pH 8.0, 100 μmoles; sodium OAA, 9.5 μmoles; *A. aerogenes* 30–50 ammonium sulfate fraction 1.4 mg; and 10 μmoles of cation (as chloride salt) indicated. Na^+ concentration, 0.021 M. The volume was 1.0 ml. The incubation was for 10 min at 30°.

TABLE VI: Inhibition of OAA Decarboxylase by Cyanide and 8-Hydroxyquinoline.

Inhibitor[a]	Concn (mM)	Pyruvate (μmoles)	Inhibn (%)
None		3.39	
KCN	1	1.85	45.4
KCN	10	1.30	61.6
8-Hydroxyquinoline	2	0.50	83.3
8-Hydroxyquinoline	10	0	100
Ethanol	4%	3.00	

[a] The reaction mixture contained: Tris-HCl buffer, pH 8.0, 100 μmoles; sodium OAA, 17.5 μmoles; *A. aerogenes* 30–50 ammonium sulfate fraction 1.4 mg; and inhibitor as indicated. The 8-hydroxyquinoline was dissolved in ethanol such that reaction mixture contained 4% ethanol, final concentration. The volume was 1.0 ml. The incubation was for 10 min at 30°.

TABLE VII: Effect of Carbon Source and Aerobiosis on Enzyme Activity.

Enzyme[a]	Carbon Source: Anaerobic, Citrate	Anaerobic, Glucose	Anaerobic, Glycerol	Aerobic, Citrate
OAA decarboxylase	0.43	0.02	0.01	0.56
Citritase	0.23	0.01	0	0.06
L-Malic enzyme (TPN)	15.5	0.03	2.5	47.2
Malate dehydrogenase	45[b]	0	2.7	354
Fumarase	6.4	0	0	28
Aconitase	20	0	0	~1
Isocitrate dehydrogenase	234	1.8	0	132
Lactate dehydrogenase	193[b]	58	73	225

[a] Values are specific activities: 100 ΔA_{340}/min per mg of protein for dehydrogenases; 100 ΔA_{240}/min per mg of protein for hydratases; and micromoles of substrate reacting per minute per milligram of protein for OAA decarboxylase and citritase. [b] Determined on 30–50 ammonium sulfate fraction.

(Korkes *et al.*, 1950) and 6.5×10^{-3} M (Harary *et al.*, 1953). Only the OAA decarboxylase of rat liver mitochondria had a lower K_m value, 1.10×10^{-4} M (Corwin, 1959). The pH optimum for OAA decarboxylase activity was 7.0–7.4.

Inhibitors of OAA Decarboxylase. Although OAA decarboxylase is not inhibited by EDTA, it is sensitive to KCN and 8-hydroxyquinoline (Table VI). Thus, 10^{-3} M KCN inhibits 45% and 2×10^{-3} M 8-hydroxyquinoline 83%. This suggests that although the decarboxylase does not require an exogenous metal for activity, it may contain a tightly bound metal prosthetic group which is essential for activity. Ouabain (10^{-3} M) did not inhibit OAA decarboxylase activity. *p*-Hydroxymercuribenzoate (5×10^{-4} M) and iodoacetamide (10^{-2} M) caused 34 and 19% inhibition, respectively.

Induction of OAA Decarboxylase. OAA decarboxylase is an inducible enzyme in this strain of *A. aerogenes* and is synthesized in large amounts when citrate is the carbon source for growth. As shown in Table VII, only traces of OAA decarboxylase activity and of citritase (the primary enzyme in the citrate fermentation) were found when the cells were grown anaerobically on glucose or glycerol in place of citrate. Five other enzymes (L-malic–TPN enzyme, L-malate dehydrogenase, *d*-isocitrate dehydrogenase, fumarase, and aconitase) were also induced by growth on citrate, and largely or completely suppressed by growth on glucose or glycerol. Interestingly, OAA decarboxylase and citritase were induced by aerobic growth on citrate, as were the other citric acid cycle enzymes save for aconitase. The failure to detect significant aconitase activity in extracts (and in whole cells) after aerobic growth on citrate strongly suggests that under aerobic conditions the pathway of citrate catabolism in the cell proceeds largely (perhaps completely) *via* cleavage to OAA and acetate (the exclusive anaerobic pathway) and not *via* the citric acid cycle. The induction of the citric acid cycle enzymes under anaerobic conditions may be only incidental (perhaps as a result of coordinate induction) since, of the citric acid cycle intermediates, only small amounts of succinate accumulated as a result of citrate fermentation (Brewer and Werkman, 1939). This succinate may arise by reduction of some OAA to L-malate before it can be decarboxylated, dehydration of L-malate to fumarate which is then reduced to succinate. A pathway of glutamate biosynthesis would also be provided by this induction process. Brewer and Werkman (1939) have previously demonstrated that significant citrate fermentative capacity by *Aerobacter* was seen only after growth on citrate and not glucose. While Dagley and Dawes (1953b) showed that in citrate–

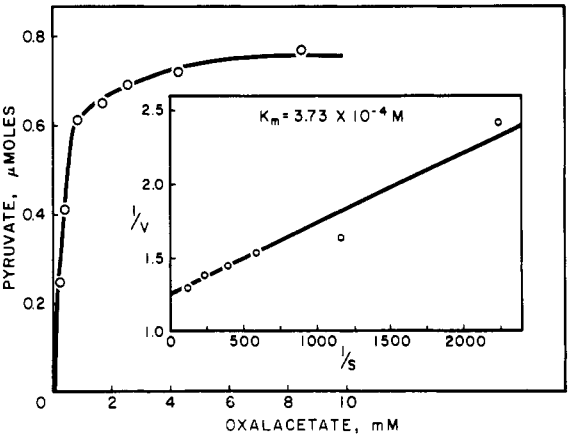

FIGURE 3: Effect of OAA concentration on decarboxylase activity. Standard assay conditions with Tris-HCl buffer (pH 8.0), 0.01 M NaCl, and disodium OAA. Enzyme was dialyzed 30–50 ammonium sulfate fraction (1.4 mg).

TABLE VIII: Effect of Carbon Source and Aerobiosis on Properties of OAA Decarboxylase.

Additions[a]	Extract of Cells Grown on (μmoles of pyruvate)		
	Glucose	Glycerol	Citrate[b]
OAA	0.92	0.13	4.98 (1.37)[c]
OAA + NaCl	0.82	0.20	4.48 (3.48)[c]
OAA + NaCl + avidin	1.0		0.40
OAA + EDTA	0.68		
Protein (mg)	2.4	2.2	1.8 (1.0)[c]
Incubation (min)	30	10	5

[a] Standard assay conditions. NaCl (10 mM), avidin (0.5 unit), and EDTA (10 mM) added where indicated. [b] Aerobic growth. [c] 30–50 ammonium sulfate fraction.

glucose mixtures, citrate in the medium was fermented only after a period of adaptation which followed the complete disappearance of glucose.

As shown in Table VIII, OAA decarboxylase activity of cells grown anaerobically on glycerol or glucose was only 1–3% of that of citrate grown cells. Moreover, this activity was no longer inhibited by avidin nor activated by Na^+ and it was now partly inhibited by EDTA. No trace of the avidin-sensitive decarboxylase was found in glucose grown cells suggesting that induction results in synthesis of a protein not previously present in the cell.

Other OAA Decarboxylases. OAA decarboxylases from the following sources were tested for avidin sensitivity with negative results: (a) extract of yeast spheroplasts at pH 8; (b) sonic extract of *L. arabinosus* cells grown in presence of L-malate and tested at pH 5.0 with Mn^{2+} (Nathan, 1961); (c) sonic extract of *M. lysodeikticus* cells (pH 7.0, McManus, 1951); and (d) washed suspensions of rat liver mitochondria (Corwin, 1959) as well as digitonin extracts of the latter at pH 7.5. Extracts of *Clostridium kluyveri* had little OAA decarboxylase activity in the absence of added ADP when tested in phosphate buffer pH 8.0, and this was unaffected by avidin. When ADP and Mg^{2+} were added, OAA decarboxylase activity was considerably increased and became sensitive to both avidin and EDTA. This activity can be attributed to reversal of the pyruvate carboxylase reaction (Scrutton and Utter, 1965), the enzyme being present in these extracts (Stern and Bambers, 1966).

Discussion

Enzymes catalyzing the irreversible decarboxylation of OAA fall into two classes: (1) the divalent-cation-dependent, EDTA-sensitive OAA decarboxylase exemplified by the prototype enzyme first discovered in *M. lysodeikticus* (Krampitz and Werkman, 1941) and (2) the OAA decarboxylase activity of L-malic enzyme in pigeon liver (Ochoa *et al.*, 1948) and in *L. arabinosus* (Korkes *et al.*, 1950) which is evident only at acid pH and which requires a divalent cation. It is clear that the OAA decarboxylase of *A. aerogenes* belongs to neither of these classes since it does not require a divalent cation and is insensitive to EDTA. The above experiments show that it is sensitive to avidin and is probably a biotinoprotein. In a future publication it will be shown that the purified enzyme does indeed contain biotin and that the enzyme is located on the ctyoplasmic membrane (G. M. Frost and J. R. Stern, unpublished experiments). It is the first example of a biotinoprotein catalyzing an irreversible decarboxylation reaction (*cf.* review of Ochoa and Kaziro, 1965) although since these experiments were performed Galivan and Allen (1967) have published an extract on the occurrence of a biotinoprotein in *Micrococcus lactilyticus* which decarboxylates methylmalonyl-CoA directly to propionyl-CoA.

Among biotinoproteins, OAA decarboxylase would appear most closely related to pyruvate carboxylase (Scrutton and Utter, 1965), a biotinoprotein that catalyzes the reversible reaction 1.

$$\text{OAA} + \text{ADP} + \text{P}_i \xrightleftharpoons{\text{Mg, acetyl-CoA}} \text{pyruvate} + \text{ATP} + \text{CO}_2 \quad (1)$$

However, OAA decarboxylase does not require ADP and P_i to catalyze OAA decarboxylation as does pyruvate carboxylase. Pyruvate carboxylase contains tightly bound manganese (Scrutton *et al.*, 1966) and in view of the sensitivity of OAA decarboxylase to cyanide and 8-hydroxyquinoline it is not excluded that the latter may also have a tightly bound metal prosthetic group. Extracts of *A. aerogenes* do not contain pyruvate carboxylase. It is also relevant that Mg^{2+} and acetyl-CoA, which specifically activate the ATP-dependent synthesis of CO_2–biotin–enzyme catalyzed by liver pyruvate carboxylase and not the carboxyl transfer from OAA to biotin–enzyme to form CO_2–biotin–enzyme (Scrutton *et al.*, 1965) are not involved in OAA decarboxylase activity.

The specific Na^+ requirement of OAA decarboxylase is quite surprising and so far unique among decarboxylases. The apparent K_m of added Na^+ was 1 mM or less. While Na^+ has been shown to activate (a) the Na^+, K^+, ATPase (Skou, 1965); (b) intestinal sucrase activity (Semenza *et al.*, 1964); (c) the depolymerization of poly-β-hydroxybutyric acid by cells of *Micrococcus halodenitrificans* (Sierra and Gibbons, 1963); (d) substrate oxidation by marine bacteria (MacLeod and Hori, 1960; and (e) the fermentation of glutamic acid by cells of *Peptococcus aerogenes* (Westlake *et al.*, 1967), the Na^+ concentrations involved (except for Na^+ activation alone of ATPase) are considerably greater. The role of Na^+ in OAA decarboxylase activity remains to be determined.

The biotinoprotein, OAA decarboxylase, is induced by growth on citrate, under both anaerobic and a erobi

conditions. It is not demonstrable after growth of *A. aerogenes* on glucose or glycerol. It obviously functions as an essential step in the conversion of citrate to pyruvate which is the real substrate for growth. Although *Streptococcus faecalis* also ferments citrate anaerobically *via* cleavage to OAA and acetate (Gillespie and Gunsalus, 1953), we find no avidin-sensitive OAA decarboxylase activity in extracts of this organism.

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References

Benziman, M., and Heller, N. (1964), *J. Bacteriol. 88*, 1678.

Brewer, C. R., and Werkman, C. H. (1939), *Enzymology 6*, 273.

Çorwin, L. M. (1959), *J. Biol. Chem. 234*, 1338.

Dagley, S., and Dawes, E. A. (1953a), *Nature 172*, 345.

Dagley, S., and Dawes, E. A. (1935b), *J. Bacteriol. 66*, 259.

Galivan, J. H., and Allen, S. G. H. (1967), *Federation Proc. 26*, 562.

Gillespie, D. C., and Gunsalus, I. C. (1953), *Bacteriol. Proc.*, 80.

Gornall, A. G., Bardawill, C. J., and David, M. M. (1949), *J. Biol. Chem. 177*, 751.

Harary, I., Korey, S. R., and Ochoa, S. (1953), *J. Biol. Chem. 203*, 595.

Herbert, D. (1951), *Symp. Soc. Exptl. Biol. 5*, 52.

Korkes, S., del Campillo, A., and Ochoa, S. (1950), *J. Biol. Chem. 187*, 891.

Krampitz, L. O., and Werkman, C. H. (1941), *Biochem. J. 35*, 595.

MacLeod, R. A., and Hori, A. (1960), *J. Bacteriol. 80*, 464.

McManus, I. R. (1951), *J. Biol. Chem. 188*, 729.

Nathan, H. A. (1961), *J. Gen. Microbiol. 25*, 415.

Ochoa, S., and Kaziro, Y. (1965), *Comp. Biochem. 16*, 210.

Ochoa, S., Mehler, A. H., and Kornberg, A. (1948), *J. Biol. Chem. 174*, 979.

Scrutton, M. C., Keech, D. B., and Utter, M. F. (1965), *J. Biol. Chem. 240*, 574.

Scrutton, M. C., and Utter, M. F. (1965), *J. Biol. Chem. 240*, 1.

Scrutton, M. C., Utter, M. F., and Mildvan, A. S. (1966), *J. Biol. Chem. 241*, 3480.

Semenza, G., Tosi, R., Vallotton-Delachaux, M. C., and Müllhaupt, E. (1964), *Biochim. Biophys. Acta 89*, 109.

Sierra, G., and Gibbons, N. E. (1963), *Can. J. Microbiol. 9*, 491.

Skou, J. C. (1965), *Physiol. Rev. 45*, 596.

Stern, J. R., and Bambers, G. (1966), *Biochemistry 5*, 1113.

Westlake, D. W. S., Horler, D. F., and McConnell, W. B. (1967), *Biochem. Biophys. Res. Commun. 26*, 461.