# THE α-KETOGLUTARIC OXIDASE SYSTEM OF AZOTOBACTER[1]

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Using cell-free preparations the terminal oxidation of carbohydrate by *Azotobacter vinelandii*, strain O, has been shown to proceed in part, if not completely, via a tricarboxylic acid cycle identical to that of animal tissue (Stone and Wilson, 1952a, 1952b). With the over-all pattern of oxidation established, a comparison between animal and bacteria of the details of the process has been started. The soluble α-ketoglutaric oxidase from pig heart catalyzes the oxidative decarboxylation of α-ketoglutarate with ferricyanide as the electron acceptor (Sanadi and Littlefield, 1951; Sanadi *et al.*, 1952) or the reduction of diphosphopyridine nucleotide by α-ketoglutarate in the presence of large amounts of coenzyme A (Sanadi and Littlefield, 1952). A succinyl-coenzyme A deacylase has been obtained from pig heart that catalyzes the breakdown of this compound (Gergely *et al.*, 1952). Reduction of diphosphopyridine nucleotide in the presence of catalytic amounts of coenzyme A was demonstrated by combining the oxidase and deacylase (Gergely *et al.*, 1952). The partially purified α-ketoglutarate oxidase system of azotobacter has been studied in a similar series of reactions.

## MATERIALS AND METHODS

Cell paste[3] obtained by the method of Stone and Wilson (1952a) was frozen at −15 C until used. Several methods were used to obtain the enzymes from the cells. Extracts from acetone powders of the cells contained very little activity. In preliminary work the Booth-Green mill was used successfully to crush the cells; however, grinding with alumina proved to be more convenient and was used routinely although much protein was adsorbed on the alumina. All the operations for the preparation of the enzymes were carried out at 0 to 5 C. Thirty g of cell paste were ground in a mortar for 3 min with 30 g of $Al_2O_3$. The alumina-cell paste was extracted with stirring for 10 min with 100 ml of 0.02 M $KHCO_3$ in 0.1 M KCl. The extract was centrifuged at 2,000 × G for 30 min to remove the cell debris and alumina, and then recentrifuged at 144,000 × G for 30 min in a Spinco centrifuge to remove the less soluble proteins. The supernate from the final centrifugation (87 ml, about 20 mg protein per ml) was brownish yellow in color and could be stored at −15 C for one week with little loss of activity.

The Spinco supernate was adjusted to pH 6 with about 2 ml of 0.2 M $KH_2PO_4$. Then 7 ml of a protamine sulfate solution (20 mg per ml, pH 5) were added slowly below the surface of a stirred solution, and the resulting flocculent precipitate was removed by centrifugation and discarded. The nucleic acid contamination as measured by the ratio of absorption at 280 mμ to that at 260 mμ (Warburg and Christian, 1941) was reduced from about 25 per cent to 5 per cent by this treatment permitting fractionation by subsequent manipulations. Precipitation with $MnCl_2$ to remove nucleic acid resulted in too great a loss of activity. Two ml of 1 M potassium phosphate pH 7 were added to the protamine supernate (90 ml, 15 mg protein per ml) and then 20 g of solid $(NH_4)_2SO_4$ were added slowly. The resulting precipitate was centrifuged and resuspended in 30 ml of cold distilled water and treated with 50 mg of calcium phosphate gel (7.9 mg per ml) (Keilin and Hartree, 1938). The suspension was centrifuged immediately and the gel discarded. One hundred mg of gel were added again to the supernate, allowed to stand 5 minutes, centrifuged, and the supernate discarded. The enzyme was eluted from the gel by dispersing in 10 ml of 0.5 M potassium phosphate pH 7.6 with a glass homogenizer, the suspension centrifuged immediately, and the gel

discarded. The enzyme solution (the oxidase) containing about 10 mg protein per ml was dialyzed for 8 to 12 hours against 0.02 M KHCO₃. The oxidase usually was stored in 0.02 M KHCO₃ pH 7.5 at −15 C; under these conditions 20 per cent of the activity was lost usually in 1 week.

The bacterial deacylase fraction present in the supernate (initial volume 90 ml) after the first $(NH_4)_2SO_4$ precipitation was precipitated with 6 g of $(NH_4)_2SO_4$ and centrifuged. The precipitate was dissolved in 20 ml of 0.1 M potassium phosphate pH 7, dialyzed, and stored as the oxidase. Little loss in activity was observed after a week's storage.

For the assay in the purification of the oxidase, the reaction with $K_3Fe(CN)_6$ as the electron acceptor, similar to that of Stumpf et al. (1947) as modified by Sanadi et al. (1952), was used. $K_3Fe(CN)_6$ (100 μM) was placed in the side arm of the Warburg vessel, and to the main chamber were added 50 μM α-ketoglutarate, 400 μM KHCO₃, 5 μM MgCl₂, 0.5 μM diphosphothiamin, 0.5 μM diphosphopyridine nucleotide, 20 mg crystalline bovine plasma albumin, and enzyme in 3.2 ml of water. The vessels were gassed for 5 minutes with $CO_2$ and equilibrated 5 minutes at 33 C; then the stopcocks were closed and the ferricyanide tipped in. The $CO_2$ evolved in the first 10 min is proportional to enzyme concentration. A unit of enzyme activity is defined as that amount which liberates 1 μM of $CO_2$ per hr, and specific activity as units per mg protein.

The reduction of diphosphopyridine nucleotide in the presence of stoichiometric amounts of coenzyme A was measured using the method of Sanadi and Littlefield (1952), and the reduction in the presence of catalytic amounts of coenzyme A by the method of Gergely et al. (1952). Details of these methods are given with figure 2 and table 2.

α-Ketoglutaric acid, obtained from Organic Specialities, was recrystallized twice from acetone and benzene. Diphosphopyridine nucleotide was supplied by Schwartz Laboratories, diphosphothiamin by Merck and Company, and crystalline bovine plasma albumin by Armour Laboratories. Purified samples of coenzyme A were kindly supplied by Drs. H. Beinert and R. W. Von Korff. α-Ketoglutarate was estimated by the method of Friedemann and Haugen (1943). Protein concentration was determined

by the biuret reaction (Gornall et al., 1949) using crystalline bovine plasma albumin as the standard. The pig heart α-ketoglutarate oxidase and succinyl-coenzyme A deacylase were prepared as detailed in Sanadi et al. (1952) and Gergely et al. (1952). Glutamic dehydrogenase was prepared from beef liver by the method of Kornberg and Pricer (1951).

### RESULTS

The results of the purification procedure for the oxidase are given in table 1. A tenfold purification ordinarily is achieved; with many preparations specific activities of 110 to 170

TABLE 1

*Fractionation of azotobacter extract for alpha-ketoglutaric oxidase*

| STAGE | SPECIFIC ACTIVITY* | PER CENT RECOVERY (TOTAL UNITS) |
|---|---|---|
| 1. Alumina extract | 8.1 | 100† |
| 2. Spinco supernate | 21 | 105 |
| 3. Protamine supernate | 21 | 80 |
| 4. Ammonium sulfate precipitate | 46 | 58 |
| 5. Eluate from calcium phosphate gel | 89 | 25 |

\* μM $CO_2$ evolved per hour per mg protein.

† Approximately 35,000 units (μM $CO_2$/hr) from the extract of 30 g cells.

have been achieved, but such higher activities were not obtained consistently. The reddish pellet obtained from the Spinco centrifugation contains a high proportion of cytochromes as shown by spectrophotometric analyses.[4] As would be expected this centrifugation destroys the ability of the supernate to oxidize substrates with oxygen as the final acceptor, though the supernate contains many soluble enzymes.

The oxidase is not pure and is associated with traces of lactic dehydrogenase, glutamic dehydrogenase, and pyruvic oxidase activity. Fractionation with neutral or alkaline $(NH_4)_2SO_4$, or refractionation with $(NH_4)_2SO_4$ did not increase further the specific activity.

A complete system for the ferricyanide assay

[4] These examinations were made by Dr. H. Mahler.

system which had a specific activity of 84 had the following activities when each of the following were omitted: enzyme, 4; α-ketoglutarate, 3; α-ketoglutarate + 10 μM succinate, 3. There is an absolute requirement for diphosphothiamin, and partial requirements of diphosphopyridine nucleotide and Mg++ (figure 1).

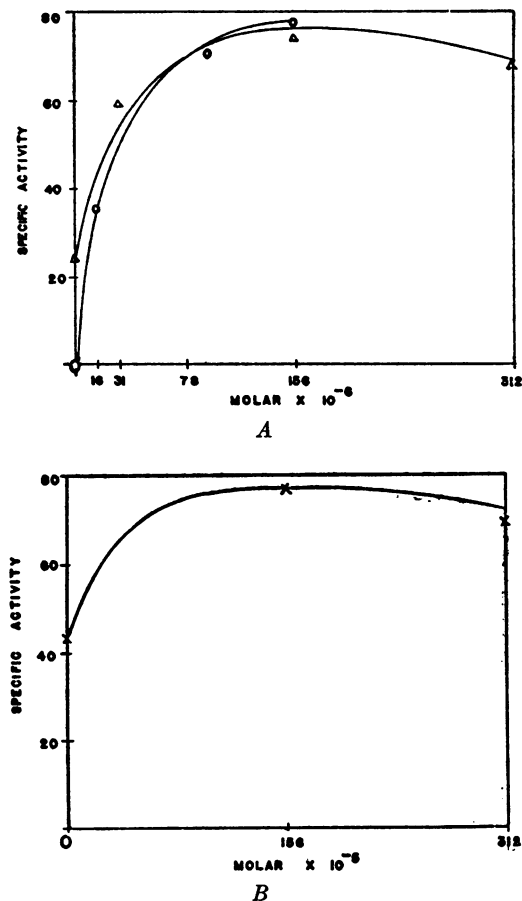quirement for phosphate could be demonstrated; arsenate has no effect. α-Lipoic acid (thiotic acid) (Brockman et al., 1952) and coenzyme A have no effect on the activity. Thiamin will not replace diphosphothiamin. The ratio of $CO_2$ to α-ketoglutarate as determined with many preparations ranged from 2.4 to 2.7. Stumpf et al. (1947) reported ratios of 3, but Sanadi et al. (1952) who also observed the lower ratios



A



B

*Figure 1.* Activation of the oxidase by added cofactors using ferricyanide as the electron acceptor. O——O = Diphosphothiamin, △——△ = Diphosphopyridine nucleotide, ✕——✕ = Mg++. For conditions of assay see text.

A small blank without enzyme is observed consistently. The albumin is added to stabilize the preparations; it has little effect on the rate during the 10 min assay period but maintains the activity over a longer period. The preparations do not metabolize succinate, and malonate has no effect on the activity. The pH optimum in the ferricyanide assay system is 6.9. No re-
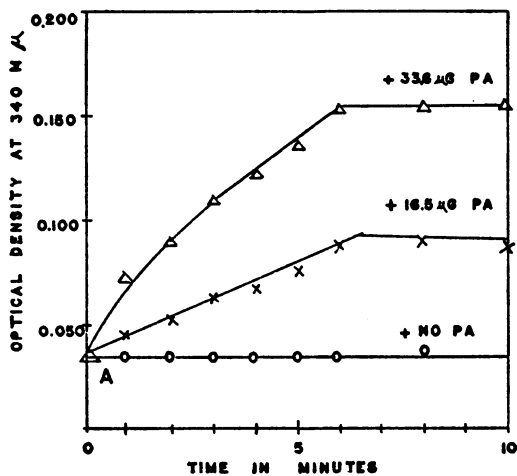


*Figure 2.* The relationship between the coenzyme A present and the diphosphopyridine nucleotide reduced by α-ketoglutarate. Conditions: 60 μM cysteine pH 7 and the amount of coenzyme A as given in μg pantothenic acid were incubated 3 min at 30 C. α-Ketoglutarate (20 μM), diphosphopyridine nucleotide (1 μM), and potassium phosphate pH 6.9 (50 μM), and water to 3.0 ml were added to the cysteine-coenzyme A mixtures. After 2 min 500 μg of oxidase were added to start the reactions (A), and the optical density change measured at 340 mμ.

with pig heart preparations have suggested the occurrence of a nonoxidative side reaction catalyzed by the oxidase, an anaerobic decarboxylation of α-ketoglutarate to succinic semialdehyde.

Measuring the reaction spectrophotometrically, figure 2 shows the direct relationship between the amount of coenzyme A added and the amount of diphosphopyridine nucleotide reduced using the oxidase alone. The change in optical density at 340 mμ is directly proportional to the coenzyme A concentration, and the reaction ceases when the coenzyme A is utilized. Reduction of diphosphopyridine nucleotide continues upon

the addition of bacterial deacylase (figure 3) as the intermediate is decomposed, and the coenzyme A liberated can react again in the oxidation. The initial jump in optical density possibly is caused by absorption by the enzyme, and the lag is caused by the reoxidation of diphospho-
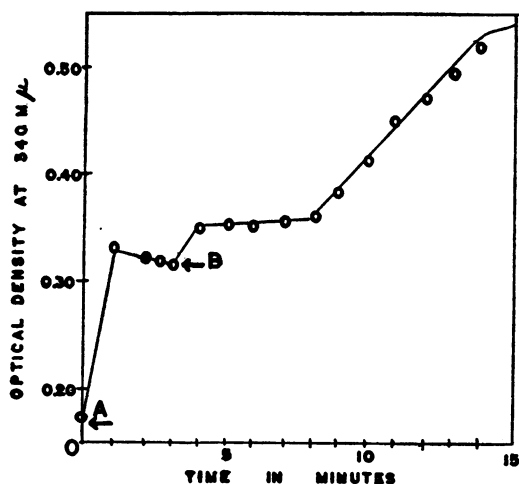


*Figure 3.* Reduction of diphosphopyridine nucleotide by $\alpha$-ketoglutarate by the bacterial oxidase alone and further diphosphopyridine nucleotide reduction upon addition of bacterial deacylase fraction. Conditions: 60 $\mu$M cysteine pH 7 added to 54 $\mu$g pantothenic acid as coenzyme A and incubated for 3 min at 30 C. $\alpha$-Ketoglutarate (20 $\mu$M), diphosphopyridine nucleotide (1 $\mu$M), MgCl$_2$ (5 $\mu$M), diphosphothiamin (0.5 $\mu$M), glycine pH 9 (100 $\mu$M), and water to 3.0 ml were added to the cysteine-coenzyme A mixture. After 2 min 150 $\mu$g of oxidase were added to start the reaction (A), and the optical density change at 340 m$\mu$ measured for 3 min. Five mg deacylase fraction added at (B).

pyridine nucleotide in the presence of $\alpha$-ketoglutarate and traces of NH$_4^+$. Glutamic dehydrogenase necessary for this reaction is present as a contaminant in both the oxidase and the deacylase fraction.

Table 2 gives a component study of the reaction with catalytic amounts of coenzyme A. This reaction can be demonstrated with the unfractionated Spinco supernate. The sum of the individual rates with the oxidase or deacylase alone is much less than the rate with the combined fractions. Coenzyme A is necessary for maximum rate of reaction, but no absolute requirement was demonstrated probably because of bound coenzyme. Mg$^{++}$ is necessary for maximal activity. Diphosphothiamin exerted only a slight stimulatory effect (20 per cent) with many of the preparations whereas in the ferricyanide reaction there is no appreciable activity without diphosphothiamin. The preparations do not catalyze the oxidation of succinate. Phosphate, arsenate, or $\alpha$-lipoic acid has no effect on the rate, nor is triphosphopyridine nucleotide reactive in this system. The data in table 3 demonstrate a cross coupling between the oxidases and deacylases obtained from pig heart and azotobacter. The reaction with catalytic amounts of coenzyme A also can be demonstrated manometrically by carrying out a dismutation in the presence of added glutamic dehydrogenase, a reaction that requires both fractions as well as diphosphopyridine nucleotide and coenzyme A for maximal activity. As no esterification of phosphorus nor formation of any compound that would form a hydroxamic acid could be demonstrated with the bacterial system, it was not investigated further.

TABLE 2

*Component study for demonstration of diphosphopyridine nucleotide reduction with azotobacter extracts*

| CONDITIONS | $\Delta D_{340}$ PER MINUTE $\times 10^{-3}$ | CONDITIONS | $\Delta D_{340}$ PER MINUTE $\times 10^{-3}$ |
|---|---|---|---|
| Complete* | 39 | $-$ Mg$^{++}$ | 20 |
| $-$ bacterial deacylase | 3 | $+$ 0.5 $\mu$M diphosphothiamin | 54 |
| $-$ bacterial oxidase | 7 | $-$ $\alpha$KG | 2 |
| $-$ CoA | 12 | $-$ $\alpha$KG $+$ 10 $\mu$M succinate | 1 |

* 100 $\mu$M glycine pH 9, 20 $\mu$M cysteine pH 7, 5 $\mu$M MgCl$_2$, 10 $\mu$M $\alpha$-ketoglutarate ($\alpha$KG), 5 $\mu$g pantothenic acid as coenzyme A (CoA), 800 $\mu$g oxidase, 3.2 mg deacylase, 1 $\mu$M diphosphopyridine nucleotide in 3.0 ml, pH 8.5. Incubated without diphosphopyridine nucleotide for 5 min at 30 C. Diphosphopyridine nucleotide added and optical density change at 340 m$\mu$ measured for 10 min.

TABLE 3

*Cross coupling between oxidases and deacylases of azotobacter and pig heart*

| CONDITIONS | $\Delta D_{340}$ PER MINUTE $\times 10^{-3}$ | CONDITIONS | $\Delta D_{340}$ PER MINUTE $\times 10^{-3}$ |
|---|---|---|---|
| Pig heart oxidase alone −800 μg | +20 | Bacterial oxidase alone −1.5 mg | −1 |
| Bacterial deacylase alone −2.4 mg | +3 | Pig heart deacylase alone −2 mg | −2 |
| Combined | +67 | Combined | +23 |

Same system as in table 2.

## DISCUSSION

The evidence presented using the reactions with diphosphopyridine nucleotide as the electron acceptor points toward succinyl-coenzyme A as an intermediate in the oxidative decarboxylation of α-ketoglutarate. Reduction of diphosphopyridine nucleotide is proportional to the amount of coenzyme A added with the oxidase used alone although they are not equivalent. This same nonequivalency was observed with the pig heart system (Sanadi and Littlefield, 1952). The pig heart deacylase has been shown specific for succinyl-coenzyme A (Gergely et al., 1952), and the pig heart oxidase has been shown to produce this compound (Sanadi et al., 1952). As these enzymes will react with their bacterial analogues, a common intermediate to all these reactions must exist, presumably succinyl-coenzyme A. The bacterial enzymes have not yet been purified to electrophoretic homogeneity as have the pig heart preparations; chemical determinations for the absolute identification of the intermediate in the bacterial system await such further purification.

Several comparisons can be made both between the pig heart and bacterial enzyme systems and between the two methods for studying α-keto-glutarate metabolism with bacterial enzymes. The bacterial oxidase is soluble while the animal enzymes must be solubilized by various methods (Sanadi and Littlefield, 1951; Kaufman, 1951). With the ferricyanide assay system the bacterial oxidase requires diphosphothiamin while the coenzyme is bound in the pig heart enzyme (Sanadi et al., 1952). There is a partial requirement for Mg⁺⁺ and diphosphopyridine nucleotide with the bacterial enzyme, but these components exert only a protective effect with the animal enzyme. As diphosphopyridine nucleotide

exerts more than a protective effect on the bacterial oxidase, the pathway of electron transport to ferricyanide may be different in the two systems. Phosphate, arsenate, α-lipoic acid, and coenzyme A have no effect in either system. The differences reflect in part the dissimilarities in degree of organization; the pig heart oxidase has a molecular weight of $4 \times 10^6$, whereas the bacterial oxidase behaves as a protein of more average molecular weight as it is not sedimented in a Spinco preparative centrifuge (144,000 × G) under conditions where the pig heart enzyme settles as a pellet. The specific activity on a mg protein basis is greater for the bacterial oxidase especially when it is remembered that the animal oxidase is assayed at 37 C while the bacterial oxidase is assayed at 33 C. This difference in specific activity may reflect the smaller molecular weight of the bacterial oxidase or possibly a difference in the turnover number. The pig heart oxidase has been purified to electrophoretic homogeneity, but the bacterial oxidase is still contaminated so the ultimate difference in specific activity may be more striking.

In the reaction of α-ketoglutarate with ferricyanide catalyzed by the oxidase, diphosphothiamin is required for activity, but coenzyme A has no effect. In diphosphopyridine nucleotide reduction either with catalytic or large amounts of coenzyme A, diphosphothiamin has little effect. The lack of coenzyme A requirement in the ferricyanide reaction is similar to the observation with the pig heart oxidase (Sanadi and Littlefield, 1952). The diphosphothiamin anomaly is difficult to explain with the available data. With the pig heart system, bound diphosphothiamin is present in the oxidase, and no requirement can be detected in any reaction.

The physiological significance of the deacylase enzymes reported here is questionable unless they play a regulatory role. In mammalian metabolism the energy of the succinyl-coenzyme A is preserved by coupled phosphorylation of adenosine diphosphate (Littlefield and Sanadi, 1952), but in azotobacter such an economy of energy has not yet been demonstrated. It should be noted that there is evidence from experiments with baker's yeast that the tricarboxylic acid cycle operates primarily to supply useful carbon skeletons for synthesis rather than energy (Krebs et al., 1952).

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### SUMMARY

A soluble $\alpha$-ketoglutaric oxidase system obtained from *Azotobacter vinelandii*, strain O, has been purified tenfold by a protamine precipitation, fractionation with ammonium sulfate, and adsorption and elution from calcium phosphate gel. Diphosphothiamin, diphosphopyridine nucleotide, and $Mg^{++}$ are required for full activity when ferricyanide is used as the electron acceptor. Added phosphate, arsenate, $\alpha$-lipoic acid, or coenzyme A is inactive in this system.

The oxidase has been shown to reduce diphosphopyridine nucleotide in the presence of large amounts of coenzyme A. Another enzyme fraction (deacylase) has been obtained from azotobacter extracts which when coupled with the bacterial oxidase reduces diphosphopyridine nucleotide in the presence of catalytic amounts of coenzyme A. The bacterial oxidase and deacylase have been shown to couple with the succinyl-coenzyme A deacylase and oxidase obtained from pig heart. This is evidence that succinyl-coenzyme A is an intermediate in the oxidation of $\alpha$-ketoglutarate by diphosphopyridine nucleotide with extracts from azotobacter.

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