TRIPHOSPHOPYRIDINE NUCLEOTIDE LINKED ISOCITRIC DEHYDROGENASE IN BACTERIA[1]

STANLEY BARBAN[2] AND SAM AJL[3]

Department of Bacteriology and Immunology, Washington University School of Medicine, St. Louis, Missouri

Received for publication March 24, 1952

The conversion of isocitric acid to *alpha*-ketoglutarate has been demonstrated both in animal tissues (Martius, 1937; Krebs and Johnson, 1937; Adler *et al.*, 1939; Ochoa, 1948) and in yeast (Kornberg and Pricer, 1951; Foulkes, 1951). Triphosphopyridine nucleotide was first implicated as a specific coenzyme for this reaction by Adler *et al.* (1939). Recently Kornberg and Pricer (1951) found in yeast two separable enzyme systems for the conversion of isocitrate to *alpha*-ketoglutarate, e.g., one triphosphopyridine nucleotide dependent and another diphosphopyridine nucleotide specific.

To date there has been no clear-cut demonstration of the occurrence of a tricarboxylic acid dehydrogenase in bacterial metabolic systems. Recently we have succeeded in obtaining from extracts of *Escherichia freundii* an ammonium sulfate fraction which reduces triphosphopyridine nucleotide spectrophotometrically in the presence of isocitric acid as substrate. *Alpha*-ketoglutarate is formed as a result of the reaction. Under the same conditions diphosphopyridine nucleotide does not function.

MATERIALS AND METHODS

Strain F920A of *Escherichia freundii*, obtained from Dr. Leland W. Parr, was employed for the isolation and partial purification of isocitric dehydrogenase. This organism has most of the characteristics of *Escherichia coli* but differs from the latter in its ability to grow on Koser's citrate agar.

To obtain large batches of cells *E. freundii* was grown, with constant aeration, at 30 C in a medium containing 1.0 per cent glucose, 0.8 per cent KH_2PO_4, 0.4 per cent $(NH_4)_2SO_4$, 0.1 per cent peptone, 0.1 per cent yeast extract, and 10 per cent tap water (for inorganic ions) at an initial pH of 7.0.

Spectrophotometric measurements of enzymic activity. The enzymatic activity of isocitric dehydrogenase was followed in the Beckman DU spectrophotometer. The enzyme preparation, diluted in 0.07 M phosphate buffer, pH 7.4, was added to the assay solution containing 0.5 ml of phosphate buffer (0.07 M) at pH 7.4,

[1] This work was supported by a research grant from the American Cancer Society on the recommendation of the Committee on Growth, National Research Council, and in part by a grant from Chas. Pfizer and Co.

[2] This paper represents a portion of a thesis submitted by S. Barban to Washington University for the degree of Doctor of Philosophy.

[3] Present address: Microbiological Chemistry Section, Department of Bacteriology, Army Medical Service Graduate School, Walter Reed Army Medical Center, Washington, D. C.

0.05 ml manganese chloride (0.01 M), 0.25 ml triphosphopyridine nucleotide (1 mg per ml), 1 ml sodium isocitrate (1 mg per ml), and distilled water to a final volume of 3.0 ml. (Details will be given in each experimental setup.) The control cuvette contained all the reagents except substrate. The changes in optical density at 340 mμ, resulting from the reduction of triphosphopyridine nucleotide, were observed at approximately one minute intervals. With this enzyme preparation no reduction of triphosphopyridine nucleotide was observed in the absence of substrate.

Determination of protein. Protein concentration of the enzymic preparation was determined colorimetrically, using the Folin phenol reagent by the method of Sutherland *et al.* (1949).

Identification of alpha-ketoglutarate. Four different methods were used for the positive identification of *alpha*-ketoglutaric acid. *Alpha*-ketoglutarate was determined and identified as the 2,4-dinitrophenylhydrazone by a modification of the method of Friedemann and Haugen (1943). The basic modification consisted of the application of the Beckman spectrophotometer for the identification of the keto acid instead of the photoelectric colorimeter.

At the end of the incubation period, the solution in the cuvette was transferred into a test tube and deproteinated with trichloroacetic acid. To the supernatant solution was added 1 ml of 2,4-dinitrophenylhydrazine reagent (1 mg of 2,4-dinitrophenylhydrazine per ml of 2 N HCl), and it was allowed to stand for 30 minutes. The hydrazone of the keto acid thus formed first was extracted into an equal volume of ethyl acetate. The hydrazone then was reextracted from the ethyl acetate into a 10 per cent sodium carbonate solution by bubbling a stream of air through a fine capillary tube extended into the bottom layer. An aliquot of the carbonate solution was pipetted into the cuvette and an equal amount of 1.5 N sodium hydroxide was added. A reddish color developed which was immediately measured spectrophotometrically at wavelengths between 400 and 600 mμ. To identify *alpha*-ketoglutarate the following procedure was employed.

Three standard solutions, each containing 20 μg per ml of either *alpha*-ketoglutarate, pyruvate, or oxalacetate, were added to three test tubes containing 2 ml of 10 per cent trichloroacetic acid and 1 ml of 2,4-dinitrophenylhydrazine reagent. These solutions were extracted first with ethyl acetate and then with a sodium carbonate solution as described above. The oxalacetate standard was kept in ice during the extraction procedure. The optical densities of the colored hydrazones were read at various wavelengths on the spectrophotometer. Three different standard absorption curves were obtained as shown in figure 1. The identity of any one of the keto acids can be established by calculating a series of ratios of optical densities at any two wavelengths of light. The standard ratios are compared then with those obtained with the unknowns.

Alpha-ketoglutarate also was identified chromatographically according to the procedure of Lugg and Overell (1948). From one large scale experiment the keto acid was identified by making the hydrazone and obtaining the melting point. In several experiments the identity of *alpha*-ketoglutarate was checked enzy-

matically in the following manner. At the end of the incubation period, the deproteinated sample was ether-extracted and chromatographed. The band corresponding to *alpha*-ketoglutarate was cut out and eluted with boiling water. This solution was oxidized now to succinate and CO_2 with acid permanganate (Ajl and Kamen, 1951). The formed succinate was identified chromatographically (Lugg and Overell, 1948) and then oxidized in the usual manner with a succinoxidase preparation obtained from beef heart (Umbreit *et al.*, 1949).

Manometric experiments. All manometric experiments were carried out in the conventional Warburg respirometer at 30 C. Resting cell suspensions were aerated for several hours to reduce endogenous metabolism.

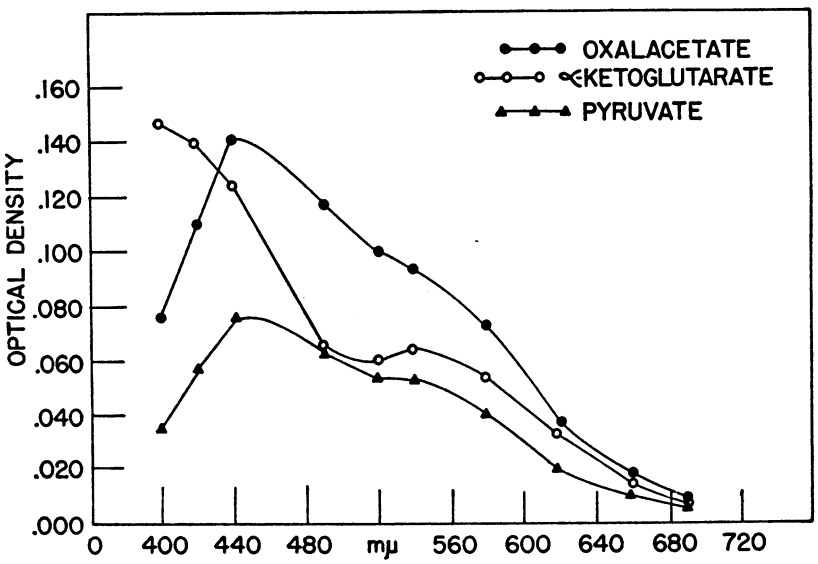


Figure 1. Standard absorption spectra of the 2,4-dinitrophenylhydrazones of oxalacetate, *alpha*-ketoglutarate, and pyruvate.

Materials. Triphosphopyridine nucleotide (~ 80 per cent pure) and diphosphopyridine nucleotide (~ 90 per cent pure) were obtained from the Sigma Chemical Company. Barium isocitrate was purchased from the Delta Chemical Company and, immediately before use, converted to the sodium salt.

EXPERIMENTAL RESULTS

Biochemical characteristics of E. freundii, strain F920A. The biochemical characteristics of *E. freundii* are essentially similar to those of *E. coli* with the exception that the former utilizes citrate as a sole source of carbon for growth. It also differs from *E. coli* in its ability to produce hydrogen sulfide.

Manometric data. Resting cells of *E. freundii* readily metabolized all of the tricarboxylic acids tested as well as *alpha*-ketoglutarate (table 1). In all cases there was an oxygen uptake with the substrates noted in table 1. Cells which

were shown manometrically to utilize readily the tricarboxylic acids were employed for subsequent isolation and partial purification of isocitric dehydrogenase.

Preparation of cell-free extract. After 24 hours' incubation at 30 C, the cells were harvested in the Sharples centrifuge and washed twice with distilled water. Seventy grams (wet weight) of cells were mixed with 140 g of powdered pyrex glass and small quantities of distilled ice water to make a paste. The chilled paste was ground by the glass cone grinding method of Kalnitsky *et al.* (1945) at 4 C. Extraction of the ground mixture was made with 70 ml of distilled water. The mixture was centrifuged in a Sorvall angle centrifuge for 3 minutes at 3,000 rpm to remove cell debris. The supernatant solution was centrifuged for 30

TABLE 1

Aerobic metabolism of tricarboxylic acids and alpha-ketoglutarate by Escherichia freundii

EXP NO.	OXYGEN UPTAKE IN μL ON			
	Citrate	*Cis*-aconitate	Isocitrate	*Alpha*-ketoglutarate
1	95	—	—	—
2	—	87	83	75

Total volume of reactants 2.3 ml. Each flask contained 0.5 ml phosphate buffer pH 7.0 (0.5 M); 50 μM of substrate; 0.5 ml of a 10 per cent suspension of cells; 0.3 ml of NaOH in center well and distilled water to volume. Temp 30 C. Time 30 minutes. Low endogenous values were subtracted.

TABLE 2

Purification of isocitric dehydrogenase from Escherichia freundii

STEP	TOTAL UNITS	SPECIFIC ACTIVITY
		units per mg protein
Crude extract	1,250	2.5
Ammonium Sulfate Fraction A	860	4.0
Ammonium Sulfate Fraction B	123	56.0
Ammonium Sulfate Fraction C	89	90.0

minutes at 4 C in the high speed head of the International Cold Centrifuge at ~ 12,000 rpm. A slightly opalescent, yellowish supernatant liquid was obtained. This extract was dialyzed for 2 hours against cold distilled water with constant stirring.

Enzyme purification. To 65 ml of the cell-free extract, 15.6 g of solid ammonium sulfate were added stepwise with mechanical stirring. After standing one hour, the mixture was centrifuged at 12,000 rpm and the precipitate discarded. Then 12.9 g of ammonium sulfate were added to the supernatant solution (Ammonium Sulfate Fraction A). The precipitate was separated from solution by centrifugation at 12,500 rpm for 15 min and discarded. To the supernatant solution (Ammonium Sulfate Fraction B) 10.5 g of ammonium sulfate were added, and after 2 hours the mixture was again centrifuged for 30 minutes. The supernatant solu-

tion was discarded and the precipitate dissolved in 0.07 M phosphate buffer (pH 7.0) to a volume of 25 ml (Ammonium Sulfate Fraction C). This fraction containing less than 1 mg of protein per ml was employed in subsequent studies. A typical protocol showing data on the specific activity of the various fractions is presented in table 2. One unit is defined as the change in log I_0/I of 0.001 per minute. The increment in density, log I_0/I, between the reading at 15 and 45 seconds after mixing of the solution, multiplied by 2, is taken as the enzymatic activity per minute. Specific activity is expressed as units per mg of protein. It is clear from the data given that the specific activity goes up with each ammonium sulfate fractionation, thus in effect purifying the enzyme.

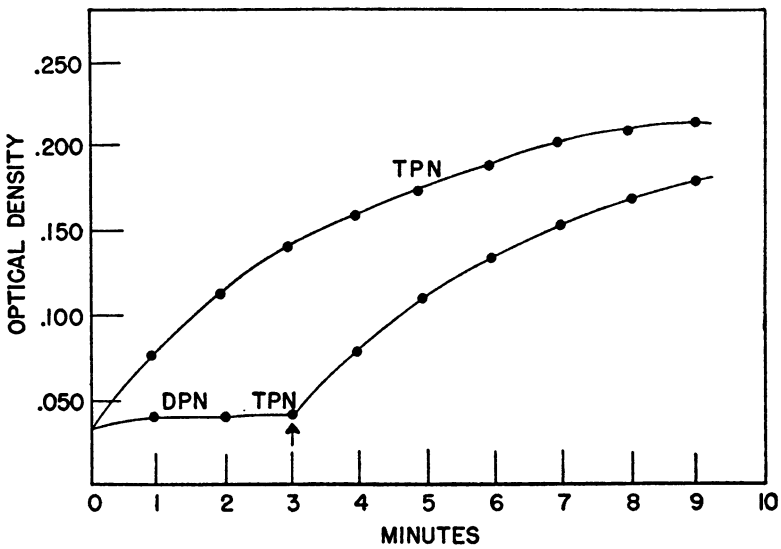


Figure 2. Pyridine coenzyme specificity for bacterial isocitric dehydrogenase. Total volume of reactants 3 ml. Each cuvette contained 0.25 ml of Ammonium Sulfate Fraction C, 0.5 ml of phosphate buffer, 0.07 M (pH 7.4), 0.05 ml of 0.01 M $MnCl_2$, and 0.25 ml of either di- or triphosphopyridine nucleotide as indicated. The experimental cuvette also contained 1 mg of sodium isocitrate. Blank cuvette lacked isocitrate.

The enzyme so prepared is relatively stable, for it can be kept for two weeks at 4 C without appreciable loss of activity. Freezing and thawing destroy the enzyme. In the procedures previously outlined, all steps therefore were conducted in the cold room at an approximate temperature of 5 C.

Pyridine coenzyme specificity. It is clear from the graph presented in figure 2 that a rapid reduction of triphosphopyridine nucleotide occurs when isocitric acid is added to the partially purified enzymic preparation described above. Diphosphopyridine nucleotide is completely inactive. The triphosphopyridine nucleotide-linked isocitric dehydrogenase is similar to the one present in yeast in that it requires no added adenosine-5-phosphate for its activity. It is clear also from the graph in figure 2 that subsequent addition of the proper coenzyme

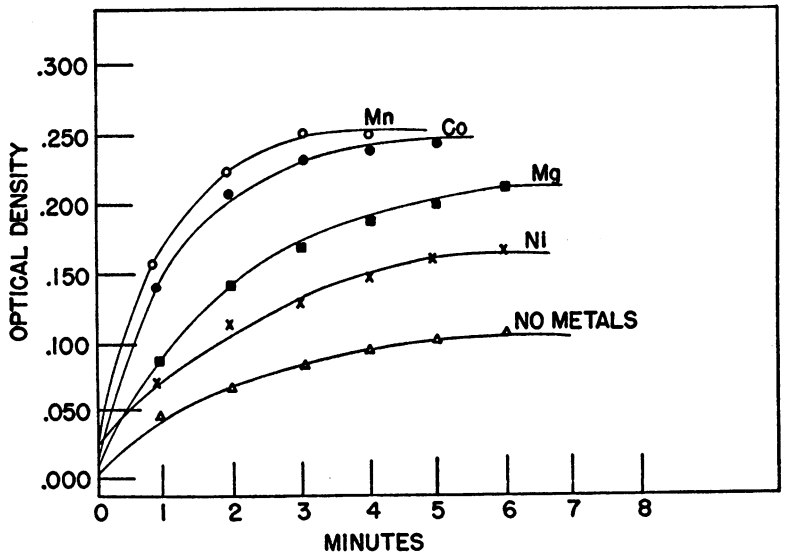


Figure 3. Metal requirements of isocitric dehydrogenase. The incubation mixture contained 0.25 ml of Ammonium Sulfate Fraction C, 0.5 ml of phosphate buffer, 0.07 M (pH 7.4), 0.25 mg of triphosphopyridine nucleotide, and 1 mg of sodium isocitrate. Where indicated 0.05 ml of either 0.01 M $MnCl_2$, $MgCl_2$, $Co(NO_3)_2 \cdot 5H_2O$, or $NiSO_4$ was added. The control cuvette contained no metal.

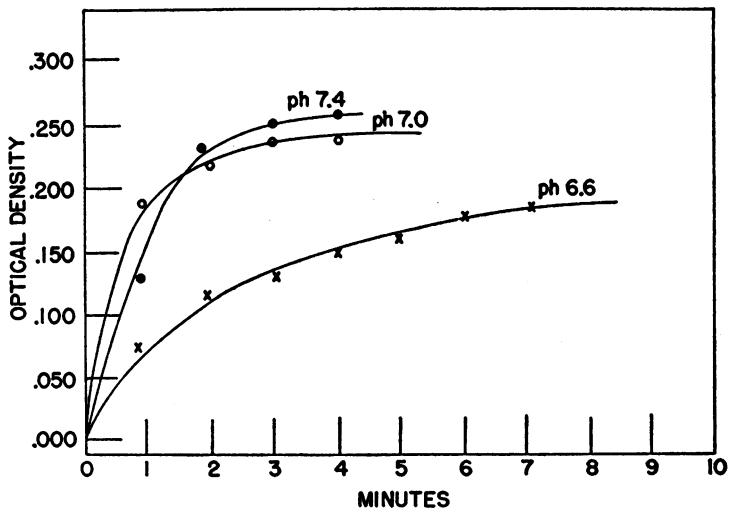


Figure 4. Effect of pH on the reduction of triphosphopyridine nucleotide by isocitric dehydrogenase. Total volume of reactants 3 ml. Each cuvette contained 0.25 ml of Ammonium Sulfate Fraction C, 0.25 mg of triphosphopyridine nucleotide, 1 mg of sodium isocitrate, 0.05 ml of 0.01 M $MnCl_2$ and 0.5 ml of phosphate buffer, 0.07 M of the indicated pH.

resulted in the expected rate of reaction, indicating no significant inhibitory effect by the inactive coenzyme present.

Inorganic metal requirement. The requirement of triphosphopyridine nucleotide isocitric dehydrogenase for metals is shown in figure 3. Mn^{++} produced a larger stimulatory effect than Mg^{++}. It is interesting that Ni^{++} is nearly as effective as Mg^{++}. Nickel has also been shown previously to be involved in another reaction of the tricarboxylic acid cycle (Ajl and Werkman, 1950). CO^{++} appears to replace the Mn^{++} requirement.

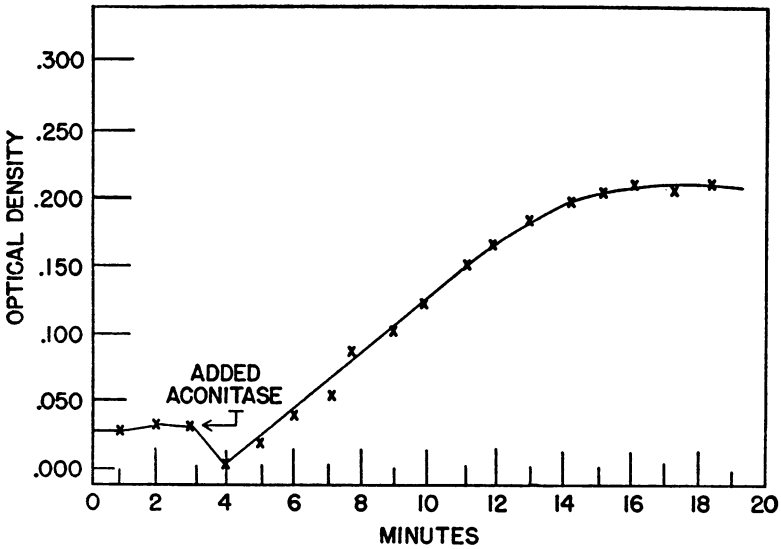


Figure 5. Optical tests with isocitric dehydrogenase and aconitase. Total volume of reactants 3 ml. Each cuvette contained 0.25 ml of Ammonium Sulfate Fraction C, 0.25 mg of triphosphopyridine nucleotide, 0.5 ml of phosphate buffer, pH 7.4 (0.07 M), 0.05 ml of 0.01 M $MnCl_2$, and 1 mg of sodium citrate. After three minutes' incubation, 0.25 ml of aconitase was added.

pH optimum. The pH optimum for the reaction is approximately 7.4 (see figure 4).

Enzyme specificity. The Ammonium Sulfate Fraction C which contains the isocitric dehydrogenase is relatively free from several other enzymes of the tricarboxylic acid cycle. It is almost completely devoid of aconitase. This was demonstrated by exposing isocitric dehydrogenase to pure citric acid and triphosphopyridine nucleotide. No reaction was observed for 3 minutes (see figure 5). On the addition of aconitase[4] (contained in a different ammonium sulfate fraction obtained from *E. freundii*), the reaction started immediately after an initial drop in optical density due to dilution of the reaction mixture. Essentially

[4] Aconitase is contained in a different ammonium sulfate fraction obtained from *E. freundii*. Its isolation and purification will be published elsewhere.

similar results were obtained with *cis*-aconitate as substrate, e.g., no reaction occurred with isocitric dehydrogenase until aconitase was added. The enzyme also was found to be inactive on *alpha*-ketoglutarate and succinate, e.g., no oxygen uptake or CO_2 evolution was noted manometrically with either compound as substrate. Neither was there any disappearance of either substrate. Spectrophotometrically no change was noted with either triphosphopyridine nucleotide or diphosphopyridine nucleotide on succinate or *alpha*-ketoglutarate.

Conversion of isocitric acid to alpha-ketoglutarate. As a result of the reduction of triphosphopyridine nucleotide, in the presence of isocitric acid, *alpha*-ketoglutarate was formed by the enzymic preparations from *E. freundii*. In table 3 are presented representative ratios of the absorption spectra of several known keto acids and that of the experimental. It is clear that the ratios of *alpha*-ketoglutarate are nearest to values obtained with the unknown. There is little doubt, therefore, that the compound formed as a result of isocitrate oxidation was indeed *alpha*-ketoglutaric acid.

TABLE 3

Identification of alpha-ketoglutarate

RATIO OF OPTICAL DENSITIES	STANDARD HYDRAZONES			EXPERIMENTAL
	Alpha-ketoglutaric acid	Pyruvic acid	Oxalacetic acid	
400:440	1.16	0.44	0.535	1.15
400:520	2.43	0.62	0.75	2.20
420:520	2.30	1.07	1.10	2.10

Alpha-ketoglutaric acid also was identified chromatographically by a procedure described by Lugg and Overell (1948) with some modification. At the end of the incubation period, the enzymic mixture was deproteinated with 6 N H_2SO_4, centrifuged, and the supernatant solution ether extracted for 48 hours. The ether solution was in turn extracted with alkaline water, evaporated to a small volume, and one half of this solution transferred on to one third of a large chromatogram sheet (Whatman filter paper no. 1) by depositing the liquid as a thin line across the top edge. One mg of known *alpha*-ketoglutarate was deposited across the second third of the sheet, and a mixture of known keto acid and the remaining one half of the solution from the experiment was deposited on the third part of the sheet. The chromatograms were developed one-dimensionally with tertiary amyl alcohol-formic acid-water mixture in the ratio of 3:1:3, respectively. After 24 hours the chromatograms were developed with bromcresol purple after previously autoclaving the sheets to remove the formic acid. The results are graphically presented in figure 6. It is readily apparent that the unknown keto acid has the identical R_f as known *alpha*-ketoglutarate and when the two are mixed together and chromatographed they exhibit the identical R_f.

To further prove the identity of *alpha*-ketoglutarate, the bands corresponding

to the keto acid were cut out and eluted with boiling water, oxidized with acid permanganate (Ajl and Kamen, 1951), ether-extracted for 24 hours, taken up in alkaline water, and rechromatographed as above. No other bands except those corresponding to succinate appeared. These in turn were eluted and exposed to succinoxidase preparations. Oxygen uptake started immediately. Nothing else,

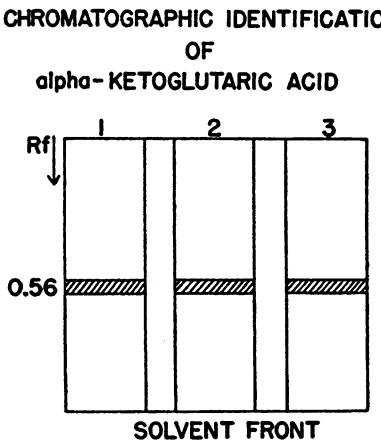


Figure 6. Chromatographic isolation and identification of the reaction product of the isocitric dehydrogenase reaction. No. 1—reaction product; no. 2—known *alpha*-ketoglutarate; no. 3—mixture of reaction product and known *alpha*-ketoglutarate.

TABLE 4

Conversion of isocitric acid to alpha-ketoglutarate

ISOCITRIC ACID ADDED	TPN* ADDED	CO_2 EVOLVED
μM	μM	μM
25	—	0.4
25	2.0	2.4
—	2.0	0.3

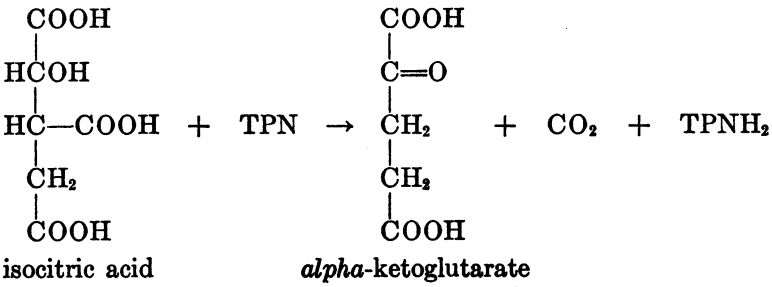
Total volume of reactants 3.0 ml. Each vessel contained 0.5 ml of phosphate buffer, pH 7.4 (0.07 M), 0.25 ml of Ammonium Sulfate Fraction C, and 0.05 ml of 0.01 M $MnCl_2$. To the appropriate vessels the following were added: 2 μM of triphosphopyridine nucleotide (calculated on the basis that the coenzyme was only ~80 per cent pure as indicated by the manufacturer), and/or 25 μM of sodium isocitrate.

* Triphosphopyridine nucleotide.

under the conditions just described, will yield succinic acid except *alpha*-ketoglutarate.

The ultimate step in the unequivocal proof for the identity of a given compound is to determine the melting point of its derivative. This was done. The unknown ketohydrazone was isolated and found to melt at ~ 220 C. On admixture of the known hydrazone of *alpha*-ketoglutarate, no drop in melting point was noted.

Stoichiometry of the reaction. The stoichiometry for the conversion of isocitric acid to *alpha*-ketoglutarate was determined manometrically. It can be shown theoretically that for every mole of triphosphopyridine nucleotide participating in the conversion of isocitric acid to *alpha*-ketoglutarate, one mole of CO_2 is produced (anaerobically). Thus:



isocitric acid *alpha*-ketoglutarate

Within experimental limit, this was found to be the case (table 4).

DISCUSSION

One of the criteria which has been widely used as evidence for the occurrence of a tricarboxylic acid cycle in many different kinds of cells has been the actual demonstration and isolation of the individual enzymes participating in the cycle. Although it is our considered opinion that the mere occurrence of such enzymes does not necessarily implicate them in any one given predetermined manner, it may, nevertheless, constitute a starting point for future investigation. It is, therefore, of considerable significance to find an isocitric dehydrogenase in those cells which actively metabolize tricarboxylic acids. In this connection, it is of significance to recall an earlier observation by Ajl and Werkman (1950) where some evidence for the intermediate formation of *alpha*-ketoglutarate as a result of citric acid breakdown by whole cells of *Aerobacter aerogenes* has been obtained.

SUMMARY

The findings reported in this paper show to our knowledge for the first time the orderly conversion of a tricarboxylic acid to *alpha*-ketoglutarate in bacteria.

The isocitric dehydrogenase from *Escherichia freundii* has been partially purified and found to be dependent upon triphosphopyridine nucleotide. Cobalt can replace the manganese requirement for this reaction.

REFERENCES

ADLER, E., VON EULER, H., GÜNTHER, G., AND PLASS, M. 1939 Isocitric dehydrogenase and glutamic acid synthesis in animal tissues. Biochem. J., **33**, 1028–1045.

AJL, S. J., AND KAMEN, M. D. 1951 Studies on the mechanism of acetate oxidation by *Escherichia coli*. J. Biol. Chem., **189**, 845–857.

AJL, S. J., AND WERKMAN, C. H. 1949 On the mechanism of carbon dioxide replacement in heterotrophic metabolism. J. Bact., **57**, 579–593.

AJL, S. J., AND WERKMAN, C. H. 1950 On the oxidative decarboxylation of *alpha*-ketoglutaric acid. Iowa State Coll. J. Sci., **24**, 279–286.

FOULKES, E. C. 1951 The occurrence of the tricarboxylic acid cycle in yeast. Biochem. J., **48**, 378–383.

FRIEDEMANN, T. E., AND HAUGEN, G. E. 1943 Pyruvic acid. II. The determination of keto acids in blood and urine. J. Biol. Chem., **147**, 415–442.

KALNITSKY, G., UTTER, M. F., AND WERKMAN, C. H. 1945 Active enzyme preparations from bacteria. J. Bact., **49**, 595–602.

KORNBERG, A., AND PRICER, W. E., JR. 1951 Di- and triphosphopyridine nucleotide isocitric dehydrogenases in yeast. J. Biol. Chem., **189**, 123–136.

KREBS, H. A., AND JOHNSON, W. A. 1937 The role of citric acid in intermediate metabolism in animal tissues. Enzymologia, **4**, 148–156.

LUGG, J. W. H., AND OVERELL, B. T. 1948 One and two dimensional partition chromatographic separations of organic acids on an inert sheet support. Australian J. Sci. Research, **1**, 98–111.

MARTIUS, C. 1937 Über den Abbau der Citronensäure. Z. physiol. Chem., **247**, 104–110.

OCHOA, S. 1948 Biosynthesis of tricarboxylic acids by carbon dioxide fixation. III. Enzymatic mechanisms. J. Biol. Chem., **174**, 133–157.

SUTHERLAND, E. W., CORI, C. F., HAYNES, R., AND OLSEN, N. S. 1949 Purification of the hyperglycemic factor from insulin and from gastric mucosa. J. Biol. Chem., **180**, 825–837.

UMBREIT, W. W., BURRIS, R. H., AND STAUFFER, J. F. 1949 *Manometric techniques and tissue metabolism.* Burgess Publishing Co., Minneapolis, Minn.