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The Enzymatic Formation of Succinic and Glyoxylic Acids From *iso*-Citric Acid in *Mycobacterium avium*

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SUMMARY

The simultaneous formation of succinic and glyoxylic acids during enzymatic breakdown of iso-citric acid was confirmed by using a purified fraction of cell free extracts of Mycobacterium avium, Takeo strain, fractionated with ammonium sulfate. This suggests that isocitritase may be active in the organism.

INTRODUCTION

An earlier publication from this laboratory reported that a substance of the same Rf as authentic succinic acid offered on the paper chromatogram as one of the reaction products when citric acid was incubated with crude exracts of Mycobacterium avium, Takeo strain. (Moriyama *et al.*, 1958). This substance was not definitely identified as succinic acid for several reasons already discussed.

In the experiments described in this paper, this substance was further studied and shown to be succinic acid. Glyoxylic acid was also recognized in the reaction mixture as the result of purification of the cell free extracts. Iso-citric acid instead of citric acid was used as substrate for production of succinic and glyoxylic acids in the later stages of purification of extracts. The results suggest that isocitritase may be active in this organism.

Methods

1. *Preparation of cell free extracts*

Crude extracts of Mycobacterium avium, Takeo strain, grown on glycerol bouillon were prepared as described previously (Moriyama *et al.*, 1958). The extracts were centrifuged at 14,500 x g for 20 minutes. The supernatant was again subjected to cetrifugation at the same gravity for the same time. The supernatant thus obtained was almost completely free from bacterial cells. All procedures were carried out between 0° and 5°C.

2. *Separation of organic acids by chromatography*

Column chromatography according to Phares *et al.* (1952) was used to separate a substance described in the introduction of this paper. The stationary and mobile phases were celite 545 (100-200 mesh) and 10 per cent butanol in chloroform respectively.

After identification of the substance by column chromatography, the paper chromatographic method of Buch *et al.* (1952) was used for demonstration of succinic acid in the reaction mixture.

3. Identification of keto acids

Keto acids were converted to 2:4-dinitrophenylhydrazone and were chromatographed on filter paper (No. 50, Toyo Roshi Co., Ltd.) following the method of El Hawary *et al.* (1953).

RESULTS

1. *Separation of succinic acid from the reaction mixture* 200 μM. potassium citrate was incubated aerobically with 10 ml. of cell free extracts at pH 7.4 (200 μM. phosphate buffer) and 37.9°C. The total volume was 34 ml. 200 μM potassium arsenite was added to the reaction mixture to inhibit succinic acid formation from α-ketoglutaric acid (Moriyama *et al.*, 1958).

After 2 hours the reaction mixture was deproteinized with 50 per cent sulfuric acid. The filtrate was extracted for 5 hours at 55°C. with ethyl ether in a Soxlhet's apparatus.

The ether was evaporated and the residue was dissolved in a small quantity of potassium hydroxide. The solution was thoroughly mixed with a sufficient amount of celite 545 and adsorbed on the latter. Then the mixture was applied to a column chromatography according to the method of Phares *et al.* (1952). The elution pattern of this chromatogram is presented in Fig. 1. There are two peaks. No peak was seen in the control run without citric acid. Peak II in Fig. 1. was shown to be α-ketoglutaric acid, by conversion to the corresponding 2 : 4-dinitrophenylhydrazone and identification by paper chromatography. The effluents which formed peak I were gathered and the solvent was evaporated. The residue was taken up in a small quantity of alkali and was rechromatographed. After the solvent had been evaporated, the properties of the residual crystals were studied. They had a rhomboid form and sublimed on heating. The melting point was 183-186°C. coinciding with authentic succinic acid.

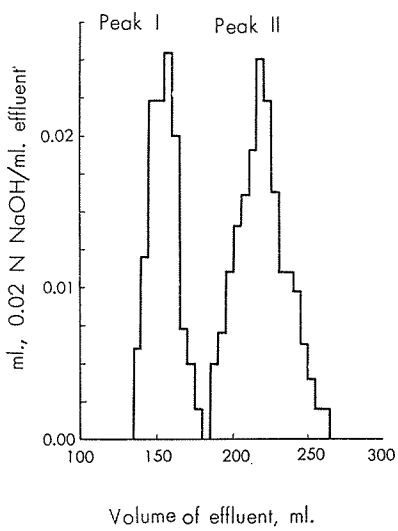


Fig. 1 Chromatographic fractionation of ethyl ether extract of reaction mixture on celite 545.

The size of the column was 1,0 × 50cm.. The mobile phase was 10% butanol in chloroform. Elution was performed at room temperature.

2. *Simultaneous occurrence of succinic and glyoxylic acids in the reaction mixture*

Cell free extracts were subjected to charcoal treatment or fractionation with ammonium sulfate. Almost no oxygen uptake was observed after these procedures with citric acid as substrate. The fraction precipitating between 45 and 60 per cent

Table 1. Effect of charcoal treatment or fractionation of cell free extracts upon enzymatic formation of succinic acid from citric acid

	Chromatographic demonstration of succinic acid in the reaction mixture
Cell free extracts	###
Cell free extracts treated with charcoal	##
0-30% $(NH_4)_2SO_4$ saturation	—
30-45% " "	+
45-60% " "	##
60-80% " "	+

Each vessels contained (in a total volume made up to 3.00 ml., with H_2O) 20 μM. potassitm citrate, 20 μM. $MgSO_4$, 20 μM. potassium arsentite, 20 μM, phosphate buffer PH 7.3 and 1,0 ml. of enzyme solution. Temperature 36.5°C.; time 60 minutes; gas phase air.

Each fraction precipitated with amonium sulfate was dissolved to half the volume of the original cell free extract and was not dialyzed.

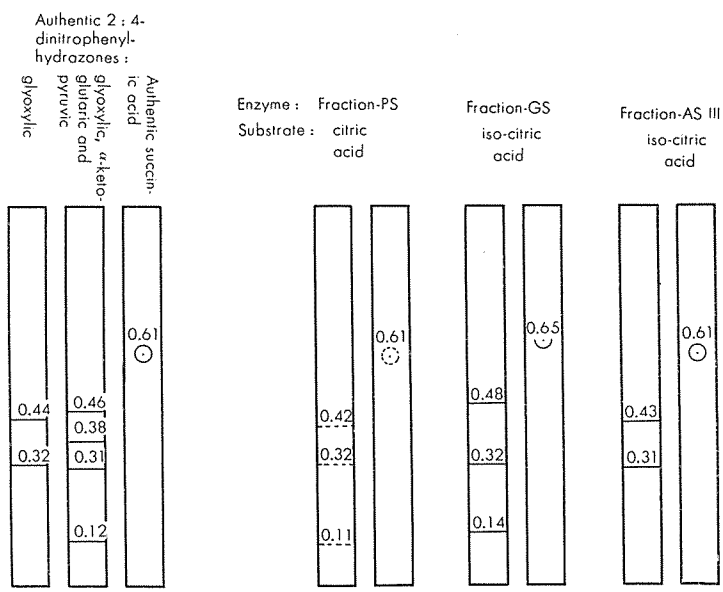
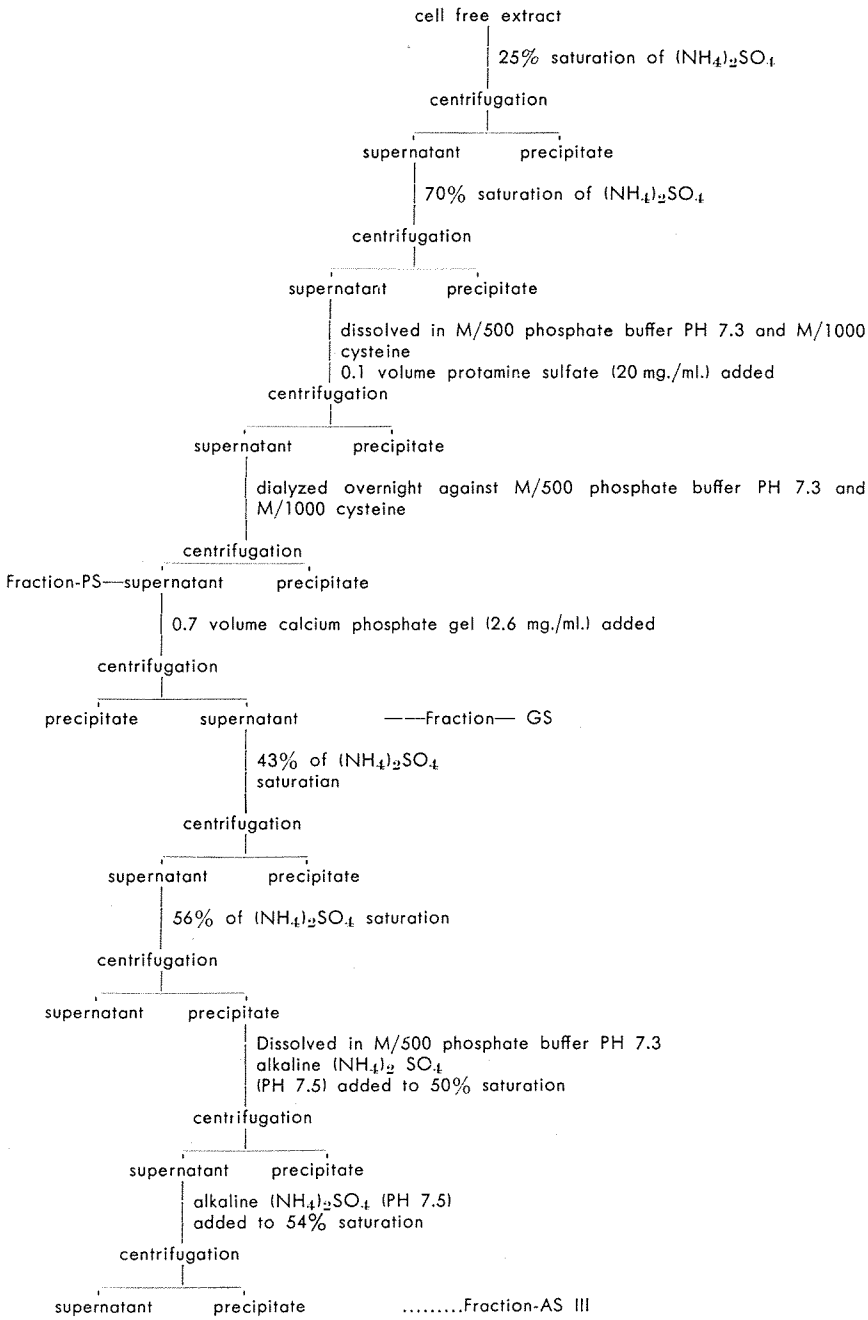


Fig. 2 Schematic chromatograms of keto acies and succinic acid

saturaion was the most active for succinic acid formation from citric acid, as shown in Table 1. After perchloric acid deproteinization, followed by filtration, neutralization and removal of potassium perchlorate, the filtrate was lyophilized and then dissolved in a small quantity of water. The insoluble residue was removed by centrifugation and a chromatographic analysis of the keto acids in the supernatant was made. Four spots were recognized as shown in Fig. 2. Two of the four spots showed Rf values corresponding to those of glyoxylic 2:4-dinitropheny-

lhydrazones. Authentic glyoxylic hydrazones showed the two spots of the trans-and cis-forms, the Rf values of which were 0.44 and 0.32. The other two hydrazones

Table 2. Fractionation of cell free extracts with ammonium sulfate



were those of α-ketoglutaric and pyruvic acids. At the same time, the presence of succinic acid in the reaction mixture was proved by paper chromatography according to the method of Buch *et al.* (1952).

Simultaneous emergence of glyoxylic and succinic acid (s) through the enzymatic degradation of citric acid provides evidence for activity of isocitritase in the strain of *M. avium*. The occurrence of this enzyme in bacteria of the genus *Pseudomonas*, many strains of *Escherichia coli*, many moulds and *Ricinus* seedling, was reviewed by Korngerg *et al.* (1957). The fraction of the cell free extract precipitating between 25 and 70 per cent satuation of ammonium sulfate was further purified according to the method used by Smith *et al.* (1957) for cell free extracts of Pseudomonas aeruginosa. The purification procedure is summarized in Table 2. All procedures were carried out at between 0° and 5°C. The presence of succinic acid and keto acids was checked by paper chromatography in each step of purification (Fig. 2). In Fraction-PS, iso-citric acid instead of citric acid served as substrate, perhaps due to removal of aconitase as the result of protamine and ealcium phosphate gel treatment.

No pyruvic acid but α-ketoglutaric acid was still demonstrated in the reaction mixture at this state. In the final state of purification glyoxylic acid was shown to be the only keto compound. The presence of succinic acid was shown at this stage of purification.

DISCUSSION

The previous studies of cirtic acid degradation in crude extract of *M. avium* (Moriyama *et al.*, 1958) were not consistent with the idea of Kusunose *et al.* (1954) that in this organism citric acid was metabolized exclusively through the tricarboxylic acid cycle. In fact, with crude cell extracts, no other keto acid except α-ketoglutaric and pyruvic acids (oxaloacetic acid when hydroxylamine was added to the reaction mixture (Nakagami, 1957)) have been proved during citric acid breakdown.

Glyoxylic acid had not been demonstrated, as cell free extracts had not been purified. Production of this acid was always accompanied by that of succinic acid, even when extracts were fractionated, as shown in Fig. 2. Fraction-GS could no longer utilize citric acid but used iso-citric acid as substrate. These facts suggest that isocitritase which breaks down iso-citric acid into succinic and glyoxylic acids may be active in this organism. The discrepancy between the results described in this paper and the results obtained by Kusunose *et al.* (1954) may be due to a difference in preparation of the enzyme.

Isocitritase activity may exist also in *M. phlei* and *M. smegmatis*, for the assay method in which citric acid and arsenite was incubated anaerobically with cell free extracts of these organisms revealed marked succinic acid formation in the reaction mixture. A biological significance for this enzyme has been suggested by Kornberg *et al.* (1957). That is, the enzyme participates in the glyoxylate cycle and plays a role in conversion of fatty acids to carbohydrates. Investigations of whether this cycle may be active in *M. avium* and of some properties of the enzyme, such as optimum pH and requirements for co-factors, are in progress.

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