ENZYMES IN *CANDIDA ALBICANS*

II. Tricarboxylic Acid Cycle and Related Enzymes

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ABSTRACT

Rao, G. Ramananda (Indian Institute of Science, Bangalore, India), M. Sirsi, and T. Ramakrishnan. Enzymes in *Candida albicans*. II. Tricarboxylic acid cycle and related enzymes. J. Bacteriol. **84**:778–783. 1962.—Evidence is presented to show the operation of the tricarboxylic acid cycle in *Candida albicans*, by studies with whole cells, cell-free preparations, and by the demonstration of most of the enzymes involved in the cycle. Cell-free extracts contained the following enzymes: condensing enzyme; aconitase; isocitric, α-ketoglutaric, succinic, and malic dehydrogenases; malic enzyme; fumarase; reduced diphosphopyridine nucleotide (DPNH) oxidase; DPNH-cytochrome *c* reductase; reduced triphosphopyridine nucleotide (TPNH) cytochrome *c* reductase; and diaphorase. Pyruvic dehydrogenase, TPNH oxidase, and transhydrogenase activities could not be detected under the test conditions.

Metabolic studies reported so far on the genus *Candida* have been mostly restricted to the ability of this organism to ferment sugars (Castellani, 1916; Benham, 1931; Hopkins and Hesseltine, 1936; Martin and Jones, 1940; Lodder and Kreger-van Rij, 1952; Thjotta and Torheim, 1955). Ward and Nickerson (1958) investigated the respiratory metabolism of normal and divisionless strains of *C. albicans*. The ability to utilize various substrates of the tricarboxylic acid cycle by *C. stellatoidea* was reported by Bradley (1958). That citrate, fumarate, malate, and succinate could serve as carbon sources for the growth of *C. utilis* and *C. krusei* was shown by Barnett and Kornberg (1960), who also reported the utilization of tricarboxylic acid cycle intermediates by *C. krusei* grown on different carbon sources. The work of Sawai (1956, 1958) has shown the presence of α-glucosidases acting on sucrose, maltose, methyl-α-glucoside and trehalose, and amylase in *C. tropicalis*. Henis and Grossowicz (1960) reported trehalase and pyruvic carboxylase in *C. albicans*. Evidence has been presented by Rao, Ramakrishnan, and Sirsi (1960) to show the operation of the Embden-Meyerhof-Parnas and hexosemonophosphate pathways in *C. albicans*.

In the absence of any report on detailed studies of the tricarboxylic acid cycle in *C. albicans*, this aspect was investigated in detail. This paper reports the utilization of tricarboxylic acid cycle intermediates by whole cells and cell-free extracts of *C. albicans*, and also the demonstration of most of the enzymes of the tricarboxylic acid cycle and electron transport chain in the organism.

MATERIALS AND METHODS

Chemicals. The following chemicals were used: ATP, adenosine triphosphate (Pabst Laboratories); DPN, DPNH, TPN, TPNH, oxidized and reduced forms of di- and triphosphopyridine nucleotides, respectively; CoA, coenzyme A; horse heart cytochrome *c*; phenazinemethosulfate; thiamine pyrophosphate; oxaloacetic acid; DL-isocitric acid lactone, lipoic acid (California Corporation for Biochemical Research); *cis*-aconitic acid (Nutritional Biochemical Corp.); citric acid, α-ketoglutaric acid, fumaric acid, sodium hydrogen malate, 2,6-dichlorophenol-indophenol (B.D.H.); sodium succinate, sodium acetate, sodium pyruvate (E. Merck, Ag.); serum bovine albumin fraction V (Armour Laboratories).

Organism. C. albicans Z 248 was used. The source and maintainance of the organism were similar to those reported earlier (Rao et al., 1960).

Medium and preparation of cell suspension.

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The medium used for growing the cells was essentially that of Bradley (1958). Cells were grown at laboratory temperature on a rotary shaker for 20 to 24 hr. This method of culture has the advantage of increased growth and lowered endogenous respiration compared with cells grown by static culture. The cells were harvested by centrifugation, washed thrice with ice-cold water, and finally suspended in water to a suitable cell concentration. The cells stored at 2 to 4 C did not show any significant loss in metabolic activity during a period of 2 to 4 days.

Cell-free extract. Cell-free extracts of the organism were prepared by grinding whole cells with three times their weight of glass powder (200 sieve) in an ice-cold mortar for 20 min. The enzymes were extracted with 0.1 M phosphate buffer (pH 6.0) and centrifuged in a Lourdes refrigerated centrifuge at 0 C at 13,000 $\times$ *g* for 30 min. The opalescent supernatant fluid was used as crude extract. When required, the crude extract was dialyzed against ice-cold water at 2 C overnight.

Dry weight determination. Dry weight was determined by evaporating a sample (5 ml) of the suspension at 105 C until constant weight was recorded.

Protein determination. The protein content of the cell-free extract was determined by the biuret method (Gornall, Bardawill, and David, 1957), using serum bovine albumin fraction V as standard.

Measurement of oxidation of substrates. Conventional manometric techniques (Umbreit, Burris, and Stauffer, 1957) were used to measure the oxidation of various substrates by whole-cell suspensions and by cell-free extracts. The central well contained 0.1 ml of 20% potassium hydroxide. Final volume, 3 ml; substrate was tipped from the side arm; temperature, 30 C; gas phase, air; shaking rate, 100 to 110 strokes per min.

Enzyme assays. The presence of condensing enzyme in cell-free extracts was shown by following the synthesis of labeled citrate from oxaloacetate and C^{14}-acetate (Ochoa, Stern, and Schneider, 1951). The incubation mixture was similar to that of Kornberg and Madsen (1958) and contained the following components (μmoles): potassium phosphate buffer (pH 7.4), 125; ATP, 10; $MgCl_2$, 10; CoA, 0.15; unlabeled potassium acetate, 10; reduced glutathione, 10; oxaloacetate, 10; C^{14}-acetate, 10 μc; cell-free extract, 2.1 mg of protein in a final volume of 1.5 ml. Incubation was at 37 C for 1 hr. Aconitase was assayed by measuring the increase in extinction values at 240 mμ with citrate (Racker, 1950); isocitric dehydrogenase by TPN reduction at 340 mμ in the presence of isocitrate (Goldman, 1956*a*); pyruvic and α-ketoglutaric dehydrogenases by measuring DPN or TPN reduction at 340 mμ or the reduction of ferricyanide at 410 mμ (Stumpf, Zarundnaya, and Green, 1947; Slater and Bonner, 1952), or by following the reduction of 2,6-dichlorophenolindophenol at 600 mμ (Kusunose et al., 1956); succinic dehydrogenase by the latter two methods; fumarase by the method of Massey (1955); malic dehydrogenase by the reduction of DPN at 340 mμ (Goldman, 1956*b*); malic enzyme by TPN reduction in the presence of manganese (Ochoa, 1955); DPNH and TPNH oxidases by following the decrease in extinction values at 340 mμ (Dolin, 1959); transhydrogenase by the method of Hochster and Katznelson (1958); diaphorase by the reduction of 2,6-dichlorophenolindophenol in the presence of DPNH or TPNH (Mahler, 1955*b*); DPNH and TPNH cytochrome *c* reductases by following the reduction of cytochrome *c* at 550 mμ in the presence of DPNH and TPNH, respectively (Horecker, 1955; Mahler, 1955*a*).

Measurement of radioactivity. After the incubation period, the reaction was stopped by adding 3 volumes of boiling alcohol (80%). The citric acid from the reaction mixture was separated by the method of Maitra and Roy (1961), using *n*-butanol–acetic acid–water (4:1:1) containing 0.05% sodium acetate to prevent the formation of acid bands on the chromatograms (Lawson and Hartley, 1958). The citric acid was detected by spraying the chromatograms (after complete drying) with 0.04% (w/v) bromcresol green in alcohol adjusted to pH 7.0; the spot corresponding to the acid was eluted with alcohol containing 0.05 N hydrochloric acid. The samples were plated on aluminium planchets and counts were recorded in a windowless gas-flow counter.

RESULTS

The utilization of various intermediates of the tricarboxylic acid cycle by whole cells and cell-free extracts of *C. albicans* is shown in Table 1. Whole cells utilized acetate and isocitrate; cell-free ex-

TABLE 1. *Utilization of intermediates of tricarboxylic acid cycle by whole cells and cell-free extracts of Candida albicans*

Substrate	Oxygen uptake*			
	Whole cells†		Cell-free extract‡	
	60 min	120 min	60 min	120 min
Pyruvate	3	5	4	13
Acetate	29	62	—	—
Citrate	2	3	—	9
cis-Aconitate	—	—	15	40
			9	20
Isocitrate	4	10	120	155
α-Ketoglutarate	3	7	12	38
Succinate	2	5	30	50
Fumarate	2	4		
Malate	1	4	3	20
Oxaloacetate	1	4		

* Oxygen uptake in μliters per mg of cells (dry wt), corrected for endogenous respiration or per mg of protein (in the case of cell-free extract).

† Each flask contained approximately 5 to 6 mg of whole cells (dry wt). All substrates were used at 75 μmoles, except isocitrate (30 μmoles).

‡ Additions (μmoles): phosphate buffer (pH 7.2), 100; DPN, 0.5; TPN, 0.35; ATP, 5; $MgSO_4$, 10; methylene blue, 30; phenazinemethosulfate, 200 μg; cell-free extract, 1.2 mg of protein. All substrates at 15 μmoles, except second reading in *cis*-aconitate (75 μmoles).

tracts utilized isocitrate, succinate, and *cis*-aconitate to a considerable extent. Incorporation of labeled acetate into citrate indicated the presence of condensing enzyme in the cell-free extract. Counts were: boiled enzyme, 490; no substrate, 500; complete system, 25,550 count/min.

Aconitase, fumarase, isocitric, α-ketoglutaric, succinic, and malic dehydrogenases, and malic enzyme are shown in Table 2. Enzymes of the electron-transport chain are shown in Table 3.

Repeated attempts to demonstrate the presence of pyruvic dehydrogenase in cell-free extract by reduction of ferricyanide, 2,6-dichlorophenolindophenol, DPN, and TPN were unsuccessful.

DISCUSSION

The poor utilization of most of the tricarboxylic acid cycle intermediates might possibly be due to the impermeability of the cell wall, brought about by the lack of corresponding permeases as has been shown in other microorganisms (Cohen and Monod, 1957; Clarke and Meadow, 1959), rather than due to the absence of the corresponding enzymes. Further, Barron, Ardao, and Hearon (1951) attributed the lack of oxidation of citric acid and succinic acid, when added to yeast as dissociated salts, to the impermeability of the yeast membrane to the salts of dicarboxylic and tricarboxylic acids. Apart from the role of permeases and physical factors like permeability, it is possible that enzymes might not be accessible to the substrates by virtue of their subcellular organization. The low metabolic activity of *C. albicans* compared with that of *C. stellatoidea* (Bradley, 1958) is probably due to species variation (King, Kawasaki, and Cheldelin, 1956). Such differences have been noted among species of the genus *Mycobacterium* (Block, 1960) and the genus *Candida* (*unpublished data*).

The increased utilization of isocitrate, succinate, and *cis*-aconitate by cell-free extracts would support the hypothesis that permeability plays an important role in the oxidation of substrates by whole cells of this organism. The inability of the cell-free extract to oxidize most of the substrates, particularly acetate, which was well utilized by the whole cells, might indicate the inefficiency of the extraction process to solubilize all the enzymes in an active state or the unsuitability of the artificial electron-transport agents used in the system. At higher substrate levels, citrate and *cis*-aconitate have shown an inhibitory effect. Citrate at 75 μmoles actually inhibited even the endogenous respiration. When this concentration was reduced to 15 μmoles, not only the inhibition was reversed but slight stimulation above the endogenous respiration was observed. A similar inhibition was recorded by Youmans, Millman, and Youmans (1956) on the utilization of these substrates by *M. tuberculosis*. The inhibition by citrate can be explained by its ability to chelate with some essential metal ions (Mudd and Burris, 1959).

Cell-free extracts showed fairly high activity of aconitase and fumarase. This might possibly be due to the fact that they are rapidly released and solubilized from the particles under mild conditions. Both of them showed a loss in activity on dialysis. Such high activity was reported by Suryanarayana Murthy, Sirsi, and Ramakrishnan (1962) in *M. tuberculosis* $H37R_V$. Among the two electron-transport agents used in detecting α-ketoglutaric and succinic dehydrogenase ac-

TABLE 2. *Enzymes of the tricarboxylic acid cycle in cell-free extracts of Candida albicans*

Enzyme*	Substrate	Measurement made	Changes in optical density
Aconitase	Citrate	Increase in extinction values at 240 mμ	0.405/30 min (crude extract) 0.300/30 min (dialized)
Fumarase	Fumarate	Fall in extinction values at 300 mμ	0.260/45 min (crude extract) 0.170/45 min (dialized)
Isocitric dehydrogenase	Isocitrate	DPN reduction at 340 mμ TPN	— 0.290/10 min
α-Ketoglutaric-dehydrogenase	α-Ketoglutarate	2,6-Dichlorophenol-indophenol reduction at 600 mμ $K_3Fe(CN)_6$ reduction at 410 mμ	0.074/30 min 0.036/60 min
Succinic dehydrogenase	Succinate	2,6-Dichlorophenol-indophenol reduction at 600 mμ $K_3Fe(CN)_6$ reduction at 410 mμ	0.174/30 min 0.030/60 min
Malic dehydrogenase	Malate	DPN reduction at 340 mμ	0.304/10 min
Malic enzyme	Malate	TPN reduction at 340 mμ	0.052/20 min
Condensing enzyme	Oxaloacetate, CoA	Radioactivity in citrate formed	25,050 count/min

* Aconitase: in 3 ml (μmoles), phosphate buffer (pH 7), 220; citrate, 30; cell-free extract, 0.8 mg of protein. Fumarase: In 3 ml, phosphate buffer (pH 7), 100; fumarate, 75; cell-free extract, 0.8 mg. of protein. Isocitric dehydrogenase: In 3 ml, tris(hydroxymethyl)aminomethane (tris) buffer (pH 7.9), 200; Mn^{++}, 20; DL-isocitrate, 3; TPN, 0.5; cell-free extract, 0.8 mg of protein. α-Ketoglutaric and succinic dehydrogenases: *2,6-dichlorophenolindophenol method.* In 3 ml, phosphate buffer (pH 7.2), 200; Mg^{++}, 10; KCN, 20; substrates, 75; 2,6-dichlorophenolindophenol (0.05%), 0.1 ml; cell-free extract, 0.47 mg of protein. *Ferricyanide method*: In 3 ml, phosphate buffer (pH 7.2), 200 ; Mg^{++}, 10 KCN, 20; potassium ferricyanide, 1; thiamine pyrophosphate, 1; CoA, 0.015; substrates, 75; cell-free extract, 0.8 mg of protein. Malic dehydrogenase: In 3 ml, glycine buffer (pH 10), 150; malate, 37; DPN, 1; cell-free extract, 0.4 mg of protein. Malic enzyme: In 3 ml, tris buffer (pH 7.2), 200; Mn^{++}, 20; malate, 75; TPN, 0.5; cell-free extract, 80 μg of protein.

TABLE 3. *Enzymes of the electron-transport chain in the cell-free extracts of Candida albicans*

Enzyme*	Substrate	Measurement made	Change in optical density
DPNH oxidase	DPNH	DPNH oxidation (340 mμ)	0.144/10 min
TPNH oxidase	TPNH	TPNH oxidation (340 mμ)	—
Transhydrogenase	TPNH and DPN	DPNH oxidation (340 mμ)	—
DPNH cytochrome *c* reductase	DPNH and cytochrome *c*	Cytochrome *c* reduction (550 mμ)	0.076/2 min
TPNH cytochrome *c*	TPNH and cytochrome *c*	Cytochrome *c* reduction (550 mμ)	0.070/2 min
Diaphorase	DPNH	2,6-Dichlorophenol-indophenol reduction (600 mμ)	0.910/8 min
	TPNH	2,6-Dichlorophenol-indophenol reduction (600 mμ)	0.570/8 min

* DPNH and TPNH oxidases and transhydrogenase: in 3 ml (μmoles), phosphate buffer (pH 7.2), 300; Mg^{++}, 10; TPNH or DPNH, 0.35; DPN, 0.5; cell-free extract, 0.32 mg of protein. DPNH cytochrome *c* and TPNH cytochrome *c* reductases: in 1 ml, phosphate buffer (pH 7.2), 70; Mg^{++}, 5; cytochrome *c*, 0.02; TPNH and DPNH, 0.66; cell-free extract, 0.28 mg of protein. Diaphorase: in 3 ml, phosphate buffer (pH 7.2), 300; Mg^{++}, 10; TPNH and DPNH, 0.66; 2,6-dichlorophenol-indophenol (0.05%), 0.1 ml; cell-free extract, 0.47 mg of protein.

tivity, 2,6-dichlorophenolindophenol appeared to be more sensitive than potassium ferricyanide. Though considerable information on the specific systems for the oxidation of DPNH in mammalian and yeast cells is available, reports on TPNH oxidases are limited. Neither TPNH oxidase nor transhydrogenase, which link TPNH to DPNH oxidase systems (Colowick et al., 1952; Kaplan et al., 1953), could be demonstrated in cell-free extracts of *C. albicans*.

These results conclusively show the operation of the tricarboxylic acid cycle in *C. albicans*.

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