The Occurrence of the Tricarboxylic Acid Cycle in Yeast

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Whereas the tricarboxylic acid cycle has been firmly established in mammalian tissues as a major pathway of tissue respiration, there is no unanimous consensus of opinion about its significance in micro-organisms (cf. Karlsson & Barker, 1948; Deffner & Franke, 1939; Clifton & Logan, 1939 on the one hand, and, for example, Oginsky, Smith & Umbreit, 1949; Baskett & Hinshelwood, 1950 on the other). Equally conflicting views are held with regard to the occurrence of a similar cycle in yeast. Citric acid formation in yeast has been observed by many workers, e.g. with whole cells in the presence of metallic ions (Wieland & Sonderhoff, 1932; Virtanen & Sundman, 1942) and in cell-free extracts fortified with adenosinetriphosphate (ATP), coenzyme A and high concentrations of Mg^{++} (Novelli & Lipmann, 1950). The oxidation of isotope-labelled acetate by yeast has been studied by Lynen (1947) and by Weinhouse & Millington (1947) who conclude that the tricarboxylic acid cycle is active in yeast.

On the other hand, Krebs (1949) believes that most of the earlier work is invalidated by the use of impure yeast. He points out further that the rates of citric and succinic (Kleinzeller, 1941) acid formations are slow when compared with the respiration, and he was unable to find evidence for the oxidation of citric and succinic acids by pure yeast. He believed that the non-oxidation of succinate, in particular, was not due to impermeability. The problem of the intermediary stages of substrate oxidation in yeast he therefore regarded as unsolved.

The present experiments arose from work upon the action of fluoroacetate on yeast, and were carried out in the hope of gaining both qualitative and quantitative information about the pathway of citric acid breakdown in enzyme preparations from pure yeast. Whilst intact cells do not metabolize added tricarboxylic acids, cell-free extracts can be obtained capable of metabolizing these acids at a rate corresponding to up to 40% of the rate of oxidation of pyruvate by the intact cells. α -Ketoglutaric acid has been identified as the end product of citrate breakdown provided that its further oxidation to oxaloacetate and pyruvate is inhibited by semicarbazide or cyanide. In contrast to the high sensitivity of animal α -keto-acid decarboxylases to arsenite and lewisite, high concentrations of these inhibitors did not affect α -ketoglutarate breakdown

in cell-free yeast extracts. The results presented establish the tricarboxylic acid cycle as a significant pathway of substrate oxidation in the strains of *Saccharomyces cerevisiae* investigated.

METHODS AND MATERIALS

Reagents. Oxaloacetic, DL-isocitric, cis-aconitic and α -ketoglutaric acids, as well as recrystallized sodium pyruvate, were prepared in this laboratory by Mr R. W. Wakelin. The pyridine nucleotides used in Exp. 2B, Table 1, were, respectively, a crude preparation from liver of coenzyme II (Co II) kindly provided by Dr Lotspeich and a sample of 60% pure coenzyme I (Co I) prepared in this laboratory. ATP was prepared from rabbit muscle by the method described by Umbreit, Burris & Stauffer (1945); its purity was 70%. Weights of ATP are expressed in mg. of Ba salt, but the latter was converted to the Na salt before use. Semicarbazide hydrochloride, KCN and sodium citrate. $2H_2O$ were British Drug Houses Ltd. reagents. All reagents were freshly made up and neutralized, if necessary, immediately before use. Phosphate buffers were mixtures of KH_2PO_4 and $Na_2HPO_4 \cdot 12H_2O$.

Analytical methods. Citrate was determined by the method of Pucher, Sherman & Vickery (1936), as modified by Buffa & Peters (1950). Steam-volatile, non-keto-acids were estimated by the method of Long (1938); HCN did not interfere in the final titration if bromothymol blue was used as indicator. The method of Friedemann & Haugen (1943) was used for the estimation of keto-acids; final readings were carried out on a Beckman spectrophotometer at 420 and 540 m μ . A correction had to be applied in the presence of semicarbazide. The 2:4-dinitrophenylhydrazones of the keto-acids were separated chromatographically (Cavallini, Frontali & Toschi, 1949) using water-saturated *n*-butanol with 2% NH_4OH as suggested by Dr R. V. Coxon (private communication). Dicarboxylic acids were separated from the tricarboxylic acids and from each other by one-dimensional ascending paper partition chromatography using Whatman no. 4 filter paper and water-saturated *n*-butanol (88%), $CHCl_3$ (10%), 98% formic acid (2%) (all v/v). The chromatograms were developed with phenol red.

Preparation of extract. The source of the enzyme was freshly bought, commercial baker's yeast (Distillers Company Ltd.). Identical results were, however, obtained with a pure strain of *S. cerevisiae* grown in the laboratory at 25° on a liquid tryptic casein-marmite-glucose medium, pH 5.0. For the preparation of the extract the yeast was washed twice on the centrifuge and then suspended in ice-cold 0.9% KCl (1:3). Disintegration was carried out in the cold room in a Mickle shaker (a cup fastened at the end of an electromagnetically operated tuning fork) with the aid of small glass beads (Ballotini 14). The suspension was then centrifuged in

the cold for 20 min. at 6000 rev./min. (7000 g). The efficiency of cell disintegration could be roughly estimated from the stratified appearance of the sediment. It must be emphasized, however, that it was not found possible to standardize fully the extent of cell disintegration, and experiments with different preparations even from the same batch of yeast are thus not strictly comparable. The cell-free extract was dialysed if required in thin collodion tubes in a rocking dialyser in the refrigerator, against 0.1 M-phosphate, pH 7.1, or 1.5% KCl as stated. All solutions were made up in glass-distilled water.

Incubation and analysis. The extracts were incubated usually in 50 ml. Erlenmeyer flasks shaken in a Warburg bath. Conventional Warburg manometers were used for anaerobic experiments. Each flask contained 2 ml. of enzyme in a final volume of 3 ml. Volumes were adjusted with 0.9% KCl. At the conclusion of the incubation the flask contents were acidified with 1 ml. of freshly prepared 25% (w/v) trichloroacetic acid. The protein precipitate was spun off, the supernatant made up to volume and suitable portions taken for analysis. All values given in the tables represent the mean of determinations on at least two duplicate flasks with a deviation from the mean of no more than $\pm 5\%$.

RESULTS

In Table 1 are summarized experiments illustrating the utilization of citrate by extracts of fresh commercial baker's yeast. It will be noted (Exp. 1) that ATP, in the presence of Mg^{++} , significantly accelerates the rate of citrate disappearance in the non-dialysed extract.

Exp. 2 shows that the marked effect of ATP + Mg^{++} consistently observed in the fresh extract is abolished on dialysis or on addition of cyanide. There always remains, however, a residual cyanide-stable system metabolizing citrate under these conditions at about two-thirds the rate in the fresh extract fortified with ATP and Mg^{++} . Once the

cyanide-sensitive, ATP-requiring system has been inactivated by dialysis, it is not reactivated by the addition of Co I or Co II in the quantities shown in Exp. 2B. The system can be reactivated, on the other hand, by the addition of a boiled extract of starved yeast (shaken 4 hr. at 37°, then spun and washed, and thereupon extracted at 90° for 5 min.) equivalent to the volume of enzyme solution used (Exp. 3). These results are corrected for the citrate present in the boiled extract. The system active in the absence of ATP is very much more stable than the cyanide-sensitive, ATP-requiring system. Even after long periods of dialysis there is no evidence that the former needs any dialysable cofactors or inorganic phosphate (cf. Table 2).

As the absence of oxygen does not completely inhibit the activity of the fresh extract, there appears to be a coupled reduction with a reducible substrate; the latter is evidently not entirely removed by even prolonged periods of dialysis. The greater inhibition of citrate disappearance observed under anaerobic conditions than in the presence of cyanide may be accounted for by the finding, in separate experiments, that 0.01 M-cyanide inhibited the endogenous oxygen uptake of fresh yeast by only about 90%. This is in agreement with the results of Dixon & Elliott (1929).

No information could be obtained on the nature of the hydrogen acceptor in the cyanide-stable system. That it is not oxaloacetate is shown by Exp. 2, Table 2, in which the keto-acid fixative cyanide did not interfere with the anaerobic citrate dissimilation; nor could any traces of malate, fumarate or succinate be discovered by chromatography. Had there been a direct split of citrate to oxaloacetate and acetate, as suggested by Deffner & Franke (1939), there

Table 1. *Citrate utilization by cell-free yeast extract*

(Each flask contained 10 μ mol. citrate, 0.08 M-phosphate buffer, pH 7.1, ATP and Mg^{++} added as shown in quantities of 1 mg. and 100 μ g., respectively. Flasks incubated at 37° for 30 min. Gas phase: air.)

Exp. no.	Extract	Additions	Citrate metabolized (μ mol.)
1	Non-dialysed	—	5.6
		ATP	6.8
		ATP + Mg	9.1
2A	Non-dialysed	—	6.6
		0.01 M-KCN	5.7
		ATP + Mg	9.1
		ATP + Mg + 0.01 M-KCN	6.8
		0.01 M-KCN	0
2B	Boiled	—	5.7
		ATP + Mg	6.2
	Dialysed 5 hr. against phosphate	ATP + Mg + Co II (50 μ g.)	6.2
		ATP + Mg + Co I (100 μ g.)	6.1
		—	7.9
3	Dialysed 4 hr. against phosphate	ATP + Mg	8.3
		ATP + Mg + boiled yeast	12.4
		juice containing 3.2 μ mol. citrate	

Table 2. *Citrate utilization in dialysed extract*

(Each flask contained 0.03M-phosphate pH 7.1 or 0.02M-veronal acetate pH 7.1 as indicated, and 10 μ mol. citrate. The N_2 was free from O_2 . Cyanide was used in a concentration of 0.01M. The flasks were incubated for 30 min. at 37°.)

Exp. no.	Age of extract (hr.)	Dialysis*	Gas phase	Additions	Citrate metabolized (μ mol.)
1	0	—	Air	Phosphate	6.0
		—	N_2	Phosphate	3.3
		—	Air	Phosphate-KCN	5.0
2	5	+	N_2	Phosphate-KCN	3.2
3	12	—	Air	Phosphate	2.2
		+	Air	Phosphate	1.6
		+	Air	Phosphate-KCN	0.9
		+	N_2	Phosphate	0.9
		+	Air	Veronal	1.8
4	24	+	Air	Phosphate	0.7
		+	Air	Veronal	0.9
		+	N_2	Phosphate	0

* Dialysis was against KCl and its duration corresponds to the age of the extract.

Table 3. *Formation of keto-acids from citric acid in dialysed extracts*

(Each flask contained 0.08M-phosphate, pH 7.1. Temp. 37°. Duration 30 min. Gas phase: air. Dialysis against 1.5% KCl, for 4 hr.)

Exp. no.	Additions	Initial concn. of citrate (μ mol.)	Citrate metabolized (μ mol.)	Keto-acid formed (μ mol.)	$\frac{E_{420}}{E_{540}}$ *	Remarks
1	—	—	—	0.9	1.53	Preparation sterile
	Citrate	6.0	3.1	2.6	1.71	
	Citrate + 0.5 mM- As_2O_3	6.0	2.6	2.7	1.64	
	Citrate + 0.1M-semicarbazide	6.0	3.2	2.9	1.83	
2	0.01M-Semicarbazide	—	—	0.3	1.86	—
	Citrate	3.0	2.1	—	1.68	—
	Citrate + 0.01M-semicarbazide	3.0	2.1	1.8	1.90	Identified as α -ketoglutarate on chromatogram
3	0.01M-KCN	—	—	0.7	1.53	Cyanhydrins split by 5 min. boiling of acidified solution
	Citrate + 0.01M-KCN	5.0	2.0	2.3	1.74	
	α -Ketoglutarate + 0.01M-KCN (boiled enzyme)	—	—	—	1.83	
				$\frac{E_{420}}{E_{540}}$		
				* Pure α -ketoglutarate	1.90	
				Pure pyruvate	1.31	

should also have occurred a formation of volatile acid; no trace, however, could be discovered in large-scale experiments on steam distillation of dialysed extracts which had metabolized 40–60 μ mol. of citric acid in the presence of 0.01M-cyanide.

Owing to the large concentration of keto-acid precursors, both in the undialysed extract and in the boiled yeast juice, attempts to estimate the keto-acid formed as a result of citrate utilization by the ATP-activated system did not yield significant results. Keto-acid roughly equivalent to the citrate metabolized was indeed formed. It was greatly diluted, however, with the pyruvate appearing even in the control, in the absence of citrate. It was not possible, therefore, to estimate accurately the E_{420}/E_{540} ratio of the hydrazone of the keto-acid formed from citrate. If, however, citrate disappear-

ance was studied in the well-dialysed extract in the presence of various keto-acid reagents, yields of α -ketoglutaric acid approaching the theoretical could be obtained (Table 3). Parallel determinations of oxygen uptakes in extracts whose blank respiration was negligible did not lead to consistent results.

The results of Exp. 1, Table 3, suggested that the further breakdown of α -ketoglutarate was not inhibited in this case by arsenite. Other experiments on the effect of arsenicals are illustrated in Table 4. It will be noted that neither arsenite nor lewisite oxide inhibit the breakdown of α -ketoglutarate in the yeast system. This result is in strong contrast to the sensitivity of the enzyme from mammalian tissue to arsenite and to lewisite (Peters, 1950). It may here be noted that yeast carboxylase is also insensitive to arsenite (Neuberg & Rosenthal, 1913).

Table 4. Breakdown of α -ketoglutarate

(Each flask contained 0.07 M-phosphate, pH 7.1, 5 μ mol. α -ketoglutarate. Duration of dialysis: 5 hr. Incubated at 37° for 30 min. Gas phase: air.)

Exp. no.	Treatment	Additions	Total keto-acid remaining (μ mol.)	$\frac{E_{420}}{E_{540}}$	α -Keto-glutarate metabolized (μ mol.)
1	Dialysed against 1.5% KCl	—	1.9	1.63	4.1
		10 μ g. Cocarboxylase	1.8	1.63	4.0
		1.0 mM-As ₂ O ₃	2.2	1.70	3.5
		Acidified control	5.0	1.96	—
2	Dialysed against 1.5% KCl	—	2.7	1.81	2.9
		0.3 mM-Lewisite oxide	2.6	1.80	3.1
	Dialysed against 0.1 M-Na ₂ HPO ₄ , then neutralized to pH 7.1	—	5.0	1.58	2.9
		20 μ g. Cocarboxylase	4.9	1.56	3.1

Table 5. Aconitase and isocitric dehydrogenase

(Each flask contained 0.08 M-phosphate, pH 7.1. 0.01 M-Semicarbazide. Enzyme dialysed 5 hr. against 1.5% KCl. Incubated at 37° for 30 min. Gas phase: air.)

Exp. no.	Additions	Citrate remaining (μ mol.)	Tricarboxylic acid metabolized (μ mol.)	Keto-acid formed (μ mol.)	$\frac{E_{420}}{E_{540}}$
1	—	—	—	0.4	1.70
	DL-isoCitrate (20 μ mol.)	—	—	2.4	1.81
	isoCitrate + 0.005 M-iodoacetate	—	—	2.4	1.95
2	—	—	—	0.5	1.62
	Citrate (5 μ mol.)	2.0	3.0	2.0	1.72
	cis-Aconitate (5 μ mol.)	2.5	2.5	2.0	1.69

Table 6. Citrate utilization by extract from pure yeast

(Each flask contained 0.08 M-phosphate, pH 7.1. Incubated at 37° for 30 min. Gas phase: air.)

Extract	Additions	Citrate metabolized (μ mol.)	Keto-acid formed (μ mol.)	$\frac{E_{420}}{E_{540}}$
Freshly prepared	Citrate (8 μ mol.) + ATP (1 mg.) + Mg ⁺⁺ (100 μ g.)	7.3	—	—
Dialysed against 1.5% KCl for 4 hr.	0.01 M-Semicarbazide	—	1.1	1.48
	Citrate (8 μ mol.) + 0.01 M-semicarbazide	3.2	3.9	1.80

Addition of cocarboxylase to the normal extract dialysed against potassium chloride had no effect. This is not surprising as yeast carboxylase, for instance, is only split at alkaline pH; the apoprotein is well known to be very unstable. Dialysis against alkaline phosphate did indeed irreversibly inactivate the further breakdown of pyruvate, without affecting the oxidation of α -ketoglutarate to oxaloacetate and pyruvate, as judged by the values of E_{420}/E_{540} shown in Table 4.

These experiments make it clear that the cyanide-stable system present in the well-dialysed extract can convert citrate to pyruvate via α -ketoglutarate. This implies the presence of aconitase, and the extract was in fact found, when tested on *cis*-aconitate or *isocitrate*, to contain sufficient enzyme to establish the aconitase equilibrium (about 90% citric acid) in less than 30 sec. under experimental

conditions. Table 5 shows in agreement with this that the behaviour of *isocitric* and *cis*-aconitic acids is identical with that of citric acid. The *isocitric* dehydrogenase of yeast was not inhibited even by high concentrations of iodoacetate in the 30 min. period of the experiment. The enzyme thus appears to differ from the iodoacetate-sensitive *isocitric* dehydrogenase described by Breusch & Keskin (1943-5) in pigeon-breast muscle preparations.

The clear demonstration of the significant stages in the tricarboxylic acid cycle in the cell-free extract from fresh baker's yeast is in contrast to the findings of Krebs (1949) with pure yeast. To eliminate any possible effect of contamination in the starting material, the essential points were confirmed upon three occasions with pure cultures of *S. cerevisiae* grown in the laboratory: Table 6 shows the results of one such experiment.

DISCUSSION

The results presented in this paper unequivocally establish the occurrence in yeast cells of various reactions of a cycle related to the tricarboxylic acid cycle as present in mammalian cells. Under optimum conditions a $q_{cit.}$ (μ l. citrate metabolized/hr./g. fresh weight at 37°) of up to -1000 has been obtained in the extract. The q_{O_2} of fresh yeast in the presence of excess pyruvate (μ l. oxygen/hr./g. fresh weight) in phosphate buffer at pH 6.2 was of the order of 5–7000. This value has been corrected for the high endogenous oxygen uptake on the assumption that added substrate does not significantly suppress the latter. Even if the true correction should be smaller (cf. Winzler, 1940), it is obvious that the rate of citrate utilization in the extract ($q_{cit.} - 1000$) could account for a significant portion of the $q_{pyr.}$ of the intact cell (μ l. pyruvate/hr./g. fresh weight = about -2500 on the assumption that the pyruvate is completely oxidized). It must be mentioned that, while the results of Novelli & Lipmann (1950) on the citrate synthesis in the cell-free extract could be confirmed, the rate of this synthesis ($q_{cit.} + 50$) in extracts from pantothenate-enriched yeast in the presence of high concentrations of magnesium was considerably lower than the rate of citrate utilization in the absence of excess magnesium. There was, however, no appreciable acetate utilization or increased oxygen uptake when a freshly prepared extract from a pantothenate-enriched yeast was incubated with acetate in the presence of ATP and cysteine. On the other hand, in the course of work on kidney by Liébecq & Peters (1949), intact yeast cells, when incubated at 37° in the presence of fumarate and fluoroacetate at pH 6.1, were observed after an initial lag phase to accumulate citrate at a rate ($q_{cit.}$ about $+700$) comparable to the rate of citrate utilization by the extract. The failure of the cell-free extract to activate acetate to any large extent does not, therefore, invalidate the view that the cycle constitutes a major pathway of oxidation in the intact cell. It is, indeed, not known whether the experimental conditions in the extract represent the optimum conditions for citrate metabolism. The pH value of 7.1 used throughout is more alkaline than the overall internal pH of the yeast cell as reported by Conway & O'Malley (1946). Repetition of citrate-utilization and α -ketoglutarate-formation experiments at pH 6 gave similar results to the experiments carried out at a more alkaline pH, but at a somewhat slower rate (about 60%).

It is assumed in this discussion that the ATP-requiring, cyanide-sensitive part of citrate breakdown also leads to α -ketoglutaric acid. That this

could not be proved has already been pointed out in the experimental section. Even if this assumption were not correct, however, it would not upset the argument that the tricarboxylic acid cycle plays a significant part in the oxidative metabolism of the cell, as a large percentage of the citrate breakdown undoubtedly leads to α -ketoglutaric acid. In this connexion it may be interesting to refer to the recent finding of Kornberg (1950) of two separate isocitric dehydrogenases in yeast both leading to α -ketoglutaric acid. They may be related to the existence of the two different pathways of citrate breakdown described in this paper.

Several other possible pathways of citrate breakdown in the dialysed extract may be excluded: chromatography did not reveal any formation of C_4 -dicarboxylic acids, nor were any steam-volatile acids formed if the reaction was stopped at the α -ketoglutarate level. Acetonedicarboxylic acid, a possible intermediate of citrate breakdown (Challenger, Subramaniam & Walker, 1927), can also be excluded: it does not give pentabromoacetone under the conditions of the citrate estimation as it is rapidly destroyed by permanganate; its 2:4-dinitrophenylhydrazones has a E_{420}/E_{540} ratio of 1.86, i.e. similar to that of α -ketoglutarate, but it is estimated to the extent of only 7% under the conditions of the Friedemann-Haugen α -keto-acid estimation. That the enzymes of the cycle operating in yeast differ from those in mammalian cells is shown by the insensitivity of α -ketoglutarate oxidation to arsenicals. The enzyme from animal tissues (Stumpf, Zarudnaya & Green, 1947) has been reported to be an aneurinpyrophosphate protein more easily dissociated, at least in acid solution, than pyruvic oxidase. The present results provide no evidence that cocarboxylase is required for the decarboxylation of α -ketoglutarate in yeast.

The existence in yeast of mitochondria containing oxidative enzymes has been shown by Chantrenne (1943–5). These are not sedimentable at the centrifugal force (7000 *g*) employed in the preparation of the extract (cf. also Nyman & Chargaff, 1949). They differ thus from the cyclophorase-containing mitochondria of kidney homogenates.

SUMMARY

1. A cell-free extract of baker's yeast rapidly metabolizes citrate. For maximum activity in the fresh non-dialysed extract adenosinetriphosphate and magnesium are required.

2. Even after thorough dialysis there remains a citrate-metabolizing system which is not affected by cyanide. Adenosinetriphosphate and magnesium are now without effect unless boiled yeast juice is also added.

3. Yields of α -ketoglutarate approaching the theoretical are obtained from citrate in the dialysed extract. The further breakdown of α -ketoglutarate to oxaloacetate and pyruvate is not inhibited by arsenicals.

4. As similar results could be obtained with a pure sample of *Saccharomyces cerevisiae* grown in the laboratory it is concluded that the tricarboxylic acid

cycle represents a significant pathway of substrate oxidation in yeast.

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Note on an Improved Method for the Preparation of Oxythiamine

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Oxythiamine (I; $R=R'=OH$) is of some interest as an antagonist of vitamin B₁ (Soodak & Cerecedo, 1944; Eusebi & Cerecedo, 1949; Daniel & Norris, 1949), more especially since it has been reported to confer significant protection in mice against the Lansing strain of the poliomyelitis virus (Jones, Foster & Henle, 1948).

It was first prepared synthetically by Bergel & Todd (1937), but this procedure is laborious and a

simple method of preparation from commercially available aneurin (thiamine, I; $R=NH_2$; $R'=OH$) is clearly desirable. Soodak & Cerecedo (1944) converted aneurin into oxythiamine in 50-70% yield by treatment with nitrous acid, but this method did not prove very satisfactory in our hands. 3-Aminopyrimidines are generally readily converted into the 3-hydroxy compounds by boiling with dilute acids and this seemed to offer an attractive simple route to oxythiamine; it was already known (Buchman & Williams, 1935; cf. Barger, Bergel & Todd, 1935) that treatment of aneurin with concentrated hydrochloric acid in a sealed tube at 150° gave chloroxythiamine (I; $R=OH$; $R'=Cl$), the side-chain hydroxyl having been replaced by chlorine and the 3-amino group by hydroxyl. Milder conditions were