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Tricarboxylic Acid Cycle Reactions in the Fungus *Zygorrhynchus moelleri*

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SUMMARY: Whole cells of the fungus *Zygorrhynchus moelleri* can oxidize acetate, *cis*-aconitate, citrate, fumarate, glucose, malate, α -ketoglutarate, pyruvate, and succinate under appropriate conditions. The rates of oxidation of acetate and succinate, in particular, are very high and exceed the rate of glucose oxidation. Diethylmalonate in high concentration inhibits the oxidation of glucose and acetate, but succinate oxidation is not greatly inhibited by malonic acid, even when the malonate concentration is twice the succinate concentration.

By freezing the cells in liquid nitrogen it is possible to investigate many of the individual reactions of the tricarboxylic acid cycle. Among the more important reactions shown were: *cis*-aconitate to citrate; citrate or *cis*-aconitate to α -ketoglutarate; α -ketoglutarate to succinate; succinate to fumarate plus malate; malate to oxalacetate plus pyruvate; and malate or pyruvate to citrate. Acetate was not metabolized. Succinate oxidation was competitively inhibited by malonate. The significance of these results with regard to the operation of the citric acid cycle in this organism is discussed.

Numerous reports have appeared in recent years dealing with the possible operation of a tricarboxylic or dicarboxylic acid cycle as the terminal respiratory mechanism in various micro-organisms (see reviews by Ajl, 1951; Krebs, Gurin & Eggleston, 1952). Among many micro-organisms so examined, bacteria have served as the main source of material, while some work has been performed on unicellular animals (Holland & Humphrey, 1953), and plants (Danforth, 1953), and on yeast (Foulkes, 1951; Krebs *et al.* 1952; Nossal, 1954). Probably, owing to the technical difficulties involved, the filamentous fungi have been relatively neglected from this point of view. Many workers have used indirect methods in order to assess the importance of such cyclic mechanisms in fungal economy (Carson, Foster, Jefferson, Phares & Anthony, 1951; Chughtai, Pearce & Walker, 1950; Halliwell & Walker, 1952; de Stevens, DeBaun & Nord, 1951).

Shu, Funk & Neish (1954), working with *Aspergillus niger* and using tracer techniques with ^{14}C -labelled glucose, have concluded that part of the citric acid formed from glucose arose from recycled C_4 -dicarboxylic acid. Garner & Koffler (1951), using the technique of simultaneous adaptation, concluded that the cycle does not exist in *Streptomyces griseus*, and that a conventional succinic dehydrogenase is present in cells grown on succinate but not in those grown on glucose. However, respiratory studies and chemical investigations of individual reactions have tended to support the supposition that a cycle might operate in *Ashbya gossypii* (Mickelson & Schuler, 1953), *Aspergillus niger* (Deffner, 1942), *Blastocladiella emersonii* (Cantino & Hyatt, 1953),

Penicillium chrysogenum (Casida & Knight, 1954; Hockenhull, Herbert, Walker, Wilkin & Winder, 1954), and *Streptomyces coelicolor* (Cochrane & Peck, 1953). The present work indicates substantially similar conclusions with *Zygorrhynchus moelleri*.

Fungal cell membranes frequently appear to be impermeable to many of the substances regarded as intermediates in the respiratory mechanisms of other organisms, and there have been few reports of attempts to overcome such difficulties by the use of non-viable cell preparations of filamentous fungi.

In the work to be reported here the phycomycete *Zygorrhynchus moelleri* has been examined for the presence of tricarboxylic acid cycle enzymes, using manometric and chemical techniques. The respiratory activities of intact cells and of a non-viable cell preparation towards citric acid cycle substrates have also been examined.

METHODS

Growth and respiration. The cultivation techniques for the organism, *Zygorrhynchus moelleri* (Commonwealth Mycological Institute No. 21994), have been described previously (Moses, 1954). This technique was again followed with the following modifications. Experiments with whole cells were performed with cells starved for 3 hr., unless otherwise stated. The crop from 22 ml. medium was harvested, washed, and resuspended in about 30 ml. phosphate buffer of the molarity and pH indicated for each experiment. 2 ml. suspension was used in each Warburg flask. The flasks were shaken for 3 hr. in order to decrease the level of the endogenous respiration before addition of the substrate. Samples were taken for dry weight and total N determinations.

For experiments with cells treated with liquid nitrogen, 40 ml. spore suspension (obtained from four agar slopes of the organism) were inoculated into four Erlenmeyer flasks (250 ml.), each containing 60 ml. medium. These were shaken in the usual way. The total crop, after washing, was suspended in about 20–25 ml. phosphate buffer of the molarity indicated and at pH 6.5, unless stated otherwise. The very thick suspension so obtained was run slowly from a wide-mouthed pipette, or added with a spoon-shaped spatula, directly into liquid nitrogen, the latter being stirred until the fungus had all been added. The suspension froze into lumps which sank to the bottom of the vessel. (Liquid oxygen has also been used but is less convenient for handling as the frozen lumps float and have a tendency to stick together.) After 30–45 min. the frozen suspension was removed and thawed at 25°. The resulting material was very heterogeneous and difficult to pipette. This was remedied by very gentle homogenization in a Potter homogenizer, the piston being rotated gently by hand and not mechanically. The volume of the resultant uniform suspension was made up to about 30 ml. with buffer, samples were taken for total N determinations, and 2 ml. samples were added to each Warburg flask. In the experiment on malic acid oxidation at two pH values, the cell suspension was made up in water to half the normal volume and 1 ml. dispensed into each Warburg flask; 1 ml. 0.16 M buffer of the required pH value was also added.

Corrections have been made for the CO_2 retained by the buffer (Moses, 1954).

Buffers were prepared by adjusting solutions of KH_2PO_4 to the desired pH value (glass electrode) with KOH or HCl .

Chromatography. (a) *Organic acids.* Samples (2 ml.) of the reaction mixture were removed from the Warburg flasks, adjusted to pH 4–4.5 with H_2SO_4 and heated to 70° . The precipitated protein was removed by centrifugation and the supernatant diluted to 50 ml. with water. The acid anions were adsorbed on Amberlite IRA-400 and eluted with 1.0 N- $(\text{NH}_4)_2\text{CO}_3$ (Bryant & Overell, 1953); all the acids were eluted by the first 20 ml. $(\text{NH}_4)_2\text{CO}_3$ solution. The eluate was evaporated to dryness over conc. H_2SO_4 *in vacuo* and left in the desiccator until no solid $(\text{NH}_4)_2\text{CO}_3$ remained. The NH_4 salts of the organic acids were dissolved in water (0.5–1.0 ml.) and a suitable volume (50–100 $\mu\text{l.}$) applied as a spot to Whatman No. 2 filter-paper. The descending chromatograms were run overnight using a solvent consisting of *tert.*-amyl alcohol (8 parts), chloroform (8 parts), water (8 parts), 98 % (v/v) formic acid (3 parts) (L. E. Bentley, personal communication). They were dried in a current of air at room temperature for some hours until all the formic acid had evaporated and were then sprayed with 0.04 % (w/v) ethanolic solution of bromocresol green containing a little NH_3 . The organic acids appeared as yellow spots on a blue background. Markers of known acids were run as their NH_4 salts: this was important since the R_f of the free acid tended to be rather higher than that of the NH_4 salt, particularly with citric and malic acids. 15 $\mu\text{g.}$ citric acid, as a spot, could be detected in this way. The acids were identified by their R_f values. Citric acid formed from malic acid was also identified by spraying the chromatogram with the Furth-Hermann reagent (Davies, 1953).

(b) *Keto-acids.* The method of formation and extraction of keto-acid hydrazones was modified from El Hawary & Thompson (1953). The reaction mixture from the Warburg flask was deproteinized by adding an equal volume of 10 % (w/v) metaphosphoric acid and allowing to stand for 1 hr. at room temperature. After centrifugation, the supernatant was treated with an excess of 2:4-dinitrophenylhydrazine in 2N-HCl for 30 min. at 37° . The hydrazones were extracted into ethyl acetate and then into 10 % (w/v) aqueous Na_2CO_3 . The latter was acidified with conc. HCl at 0° , the hydrazones again extracted into ethyl acetate and the solution evaporated to dryness in a stream of cold air. The dry hydrazones were dissolved in a little 6 % (w/v) aqueous NH_3 and applied to Whatman No. 3 filter-paper as spots or bands. Descending chromatograms were run overnight using *n*-butanol saturated with 6 % (w/v) aqueous NH_3 as solvent (Fincham, 1953), the keto-acid hydrazones being identified by their R_f values and by reduction to the corresponding amino acid. Known keto-acid 2:4-dinitrophenylhydrazones were run as markers.

The keto-acid chromatogram was dried in a current of air at room temperature, the spot cut out, and macerated in 6 % (w/v) aqueous NH_3 . After 1 hr. the suspension was centrifuged, the supernatant acidified with conc. HCl at 0° and extracted with ethyl acetate. The latter was evaporated to dryness in a current of air at room temperature. The hydrazones were dissolved or

suspended in about 1 ml. 0.5–1.0N-CH₃COOH, Adam's catalyst added, and the mixture treated with H₂ at a pressure of 50 lb./sq.in. for 15–30 min. until the yellow colour had disappeared (Towers, Thompson & Steward, 1954). The solution of amino acids was evaporated to dryness *in vacuo* over conc. H₂SO₄. The amino acids were redissolved in a suitable volume of water and applied to Whatman No. 3 or 4 filter-paper for one-dimensional chromatograms using as solvents either 75% (v/v) phenol, 25% (v/v) water in an atmosphere of NH₃, or 40% (v/v) *n*-butanol, 50% (v/v) water, 10% (v/v) glacial acetic acid. After drying at 80–90°, and spraying with ninhydrin, the amino acids were identified by their *R_f* values and by comparison with known markers run on the same chromatogram. Two-dimensional chromatograms were run with phenol-NH₃ in one direction and collidine-lutidine in the other, the amino acids being identified from the map given by Dent (1948); known amino acids were incorporated as markers.

Chemicals. All chemicals were commercial products except oxalacetic acid 2:4-dinitrophenylhydrazone which was a gift from the Biochemistry Department, University College, London. Armour Laboratories' 'Liver Coenzyme Concentrate' was used as a source of coenzyme A and of di- and triphosphopyridinenucleotides.

Acid substrates were used as their Na salts, and solutions were adjusted to a definite pH value before use.

Dry weight and total nitrogen determinations. Dry weights were obtained by filtering portions of cell suspensions through weighed sintered glass crucibles and drying at 105–110° to constant weight. Total N determinations were performed by the standard micro-Kjeldahl technique, using a selenium catalyst during the digestion process which was continued for 8 hr. after the solution had cleared.

RESULTS

Respiration of intact cells

The ability of intact unstarved cells to oxidize tricarboxylic acid cycle intermediates, acetate and glucose was examined. At high pH values (6.8) only glucose and acetate stimulated the rate of oxygen uptake (Table 1). When the pH value was lowered to about 3.4 all the intermediates tested were oxidized (Table 1), though now acetate was inhibitory even to the endogenous respiration (Moses, 1955*b*). The presence of permeability barriers to weak acid substrates is well known in micro-organisms, and attempts have been made to overcome them either by working at low pH values so as to suppress the ionization of the weak acids, or by the use of cell-free extracts. Krebs *et al.* (1952) found, however, that with baker's yeast a low pH value was ineffective in removing the permeability barrier to these acids.

The absolute rates of oxidation of the various substrates by *Zygorrhynchus moelleri* varied considerably from preparation to preparation, although the relative order remained the same. For example, the Q_{o₂} (succinate) has varied from 40 to 105; the values given in Table 1 are typical.

Table 1. *Rates of oxidation of tricarboxylic acid cycle intermediates, glucose, and acetate*

Warburg flasks contained in a total volume of 3.0 ml.: 5.76 mg. dry wt. living cells (0.53 mg. cell N), 0.1 M-K phosphate buffer of the pH indicated, and 0.02 M-substrate. Centre wells contained 0.2 ml. of 15 % (w/v) KOH. Gas phase air; temp. 25°. Final max. rates of O₂ uptake recorded.

Substrate	pH	Q _{O₂}	q _{O₂} (N)
None (endogenous)	3.4	10.4	113.6
<i>cis</i> -Aconitate	3.4	13.8	151.6
Citrate	3.4	28.4	310.8
Fumarate	3.4	53.2	579.8
Glucose	3.4	68.0	742.8
L-Malate	3.4	37.6	409.2
α -Ketoglutarate	3.4	51.4	560.8
Pyruvate	3.4	59.0	644.2
Succinate	3.4	70.4	769.2
None (endogenous)	6.8	16.2	177.2
Acetate	6.8	125.6	1371.6
Glucose	6.8	92.4	1008.0

By the oxidation of small, known, quantities of these acids and measurement of the total oxygen uptake and carbon dioxide evolution in excess of the endogenous respiration, assuming the latter to continue during the respiration of external substrates (Moses, 1955*a*), the degrees of total oxidation and R.Q.'s were determined (Table 2). A lag was observed in the oxidation of all these substrates, particularly citrate, affecting both the oxygen uptake and carbon dioxide output (Fig. 1).

Table 2. *Degree of total oxidation and R.Q. for tricarboxylic acid cycle intermediates*

Warburg flasks contained in a total volume of 3.0 ml.: 3.72 mg. dry wt. cells, 0.067 M-phosphate buffer, pH 3.4, and 3 μ moles of each substrate (6 μ moles of DL-malate). Centre wells contained 0.2 ml. of 15 % (w/v) KOH where necessary. Gas phase air; temp. 25°. Values reported are corrected for the endogenous gas exchange.

	Substrate				
	Citrate	Fumarate	Glucose	DL-Malate	Succinate
μ mole O ₂ absorbed/ μ mole substrate:	1.52	1.07	2.17	1.40	1.49
μ mole CO ₂ evolved/ μ mole substrate:	3.20	2.09	2.41	2.52	2.14
R.Q.:	2.11	1.95	1.11	1.80	1.44
Theoretical R.Q. for complete oxidation:	1.33	1.33	1.00	1.33	1.14
Degree of total oxidation based on CO ₂ evolution (%):	53.3	52.5	40.2	63.1	53.6

Total endogenous O₂ uptake during experiment: 10.7 μ mole.

Total endogenous CO₂ evolution during experiment: 9.4 μ mole.

R.Q. (endogenous) = 0.88.

The action of malonate on the ability of the cells to oxidize succinate was investigated (Table 3). The malonate concentration necessary to produce inhibition was relatively high; the degree of inhibition was maximal im-

mediately after the addition of malonate, decreasing as the experiment proceeded. Even when the malonate concentration was twice that of the succinate the degree of inhibition was small. As subsequent work showed that succinoxidase in this organism is competitively inhibited by malonate in the usual way, it is interesting to note the ability of the cell to protect its enzymic

Fig. 1. Gas exchange of intact cells metabolizing various substrates. Experimental details as Table 2. Substrate added at time zero; endogenous gas exchanges deducted. A, glucose; B, succinate; C, fumarate; D, DL-malate; E, citrate. (a) Oxygen uptake; (b) carbon dioxide evolution.

Table 3. *Inhibition of succinate oxidation by malonate in whole cells*

Warburg flasks contained in a total volume of 3.0 ml.: 2.8 mg. dry wt. cells, 0.067 M-phosphate buffer, pH 3.4, and, where added, 0.01 M-succinate and malonate as indicated. Centre wells contained 0.2 ml. of 15% (w/v) KOH. Gas phase air; temp. 25°. Malonate added to the cells simultaneously with the succinate. Endogenous O₂ uptake subtracted.

Malonate concn. (M) ...	0.0	0.02	0.016	0.01	0.006	0.002
Succinate concentration.	—	0.5	0.625	1.00	1.67	5.00
Malonate concentration.	—	—	—	—	—	—
O ₂ uptake (μl.) in 270 min.	642	474	483	740	620	610
Inhibition (%)	—	26.2	24.7	—15.3	3.4	5.3
Final rate of O ₂ uptake (μl./hr./flask)	200	180	177	230	200	200
Inhibition (%)	—	10.0	11.5	—15.0	0.0	0.0

Total endogenous O₂ uptake in 270 min.: 268 μl.

Final endogenous rate of O₂ uptake: 30 μl./hr./flask.

apparatus against inhibition even when it seems probable that the malonic acid was able to enter the cells.

Beevers, Goldschmidt & Koffler (1952) have suggested the use of esters of weak acids to overcome permeability barriers at high pH values. As acetate was oxidized by *Zygorrhynchus moelleri* at high pH values only, and malonic acid was active at low pH values only, the action of diethylmalonate on acetate and glucose oxidation was investigated, and compared with the effect of a quantity of ethanol equivalent to that present in the ester. Considerable inhibition could be produced by high concentrations of the ester, though lower concentrations were inactive or even stimulatory. Acetate oxidation was more sensitive to the ester than was glucose oxidation; ethanol was virtually without effect. (Table 4). The latter observation indicates that effects due to the ester may be attributed to the acid moiety of the molecule and not the alcohol.

Table 4. *Inhibition of glucose and acetate oxidation by diethylmalonate*

Warburg flasks contained in a total volume of 3.0 ml.: 4.3 mg. dry wt. cells starved in buffer for 24 hr., 0.067 M-phosphate buffer, pH 6.8, and, where added, 0.022 M-glucose, 0.067 M-acetate, and diethylmalonate and ethanol as indicated below. 0.2 ml. of 15% (w/v) KOH in centre wells. Gas phase air; temp. 25°. Endogenous values not subtracted.

	Glucose oxidation				Acetate oxidation			
	0.00	0.10	0.03	0.01	0.00	0.10	0.03	0.01
Diethylmalonate concn. (M)								
O ₂ uptake before addition of ester (μl./hr./flask)	165	165	165	165	293	293	293	293
O ₂ uptake after addition of ester (μl./hr./flask)	—	6	116	246	—	5	72	264
Inhibition (%)	—	96.4	29.7	—49.1	—	98.3	75.5	9.9
Ethanol concn. (M)								
O ₂ uptake before addition of ethanol (μl./hr./flask)	165	165	165	165	293	293	293	293
O ₂ uptake after addition of ethanol (μl./hr./flask)	—	175	170	170	—	260	260	260
Inhibition (%)	—	—6.1	—3.0	—3.0	—	11.3	11.3	11.3

This work with intact cells suggested that whatever systems might be present in the fungus for the oxidation of organic acids, the reactions were very well integrated (as evidenced by the high rates of respiration) and well protected against inhibitors. The prospects of isolating any individual reactions in such cells did not appear to be good, and it was considered desirable to attempt to find a cell-free preparation capable of respiring tricarboxylic acid cycle intermediates similar to those metabolized by whole cells.

Non-viable cell preparations

Various techniques for overcoming the permeability difficulties encountered with whole cells were investigated. The technique of treatment with liquid nitrogen (Lynen & Neciullah, 1939) finally adopted gave preparations as active as any tried and was easy to carry out.

The methods investigated included: drying of the cells *in vacuo* over various desiccants; freeze-drying; acetone powdering; disruption in a high-pressure

apparatus (Milner, Lawrence & French, 1950; French & Milner, 1951); disruption in a bacterial press (Hughes, 1951); grinding in a cold mortar with sand and either 0.4 M-sucrose or mannitol, or the alkaline isotonic potassium chloride solution of Potter (1948); treatment with solid carbon dioxide ('dricold') (Krebs *et al.* 1952). Methods involving drying generally resulted in inactive preparations; those involving disruption produced active preparations, though the presses were inconvenient to use; grinding by hand was very tedious and the percentage of cells disrupted quite small even after some 10 min. Grinding in a Potter tissue homogenizer or disruption in a Waring blender were ineffective. 'Dricold' was less effective than liquid nitrogen, probably because the rate of cooling of cells deposited on the 'dricold' in a thin plastic tube was much less than when the cell suspension was added directly to liquid nitrogen. Krebs *et al.* (1952) were able to pack alternate layers of 'dricold' and crumbled commercial baker's yeast obtained from a block. Good contact was thus obtained between the yeast and the 'dricold' and the cooling was consequently rapid. *Zygorrhynchus moelleri* could not be obtained in a similar state so that the contact was inferior and the rate of cooling less.

Attempts were made to obtain a mitochondrial preparation from the organism. After grinding in a cold mortar, the remaining complete cells, cell debris, and sand were centrifuged at 2000 g. The supernatant fluid was centrifuged for 15 min. at 10,000 g. A pellet was obtained of approximately uniformly sized particles which stained with tetrazolium chloride and Janus Black in the manner of mitochondria. The preparation showed almost no oxidative activity, due perhaps to an unavoidable rise in temperature to 10–12° during centrifugation. As a refrigerated centrifuge was not available the technique was not pursued further.

Cells treated with liquid nitrogen were used in the following experiments in which several individual steps in the tricarboxylic acid cycle were investigated.

Succinate oxidation

Succinate was oxidized with a small evolution of carbon dioxide (Table 5). Chromatography of the products demonstrated the formation of fumarate and malate. Although succinate was oxidized by the preparation more rapidly than any other substrate tested, the rate of oxidation was only about 4% of that by whole cells (cf. Table 1).

Table 5. *Oxidation of succinate by liquid nitrogen-treated cells*

Warburg flasks contained in a total volume of 2.5 ml.: 5.30 mg. cell N, 0.067 M-phosphate buffer, pH 6.5, and, where added, 0.0625 M-succinate. For measurement of the O₂ uptake the centre wells contained 0.2 ml. of 15% (w/v) KOH. Gas phase air; temp. 25°.

	Endogenous	With succinate	
		Uncorrected	Minus endogenous
$q_{O_2}(N)$ (30–90 min.)	4.0	27.9	23.9
$q_{CO_2}(N)$ (30–90 min.)	3.5	5.6	2.1
R.Q.	0.88	0.20	0.09

Effect of malonate on succinate oxidation. Succinate was oxidized in the presence of various concentrations of malonate. Using the formula (Krebs *et al.* 1952):

$$\frac{1-n}{n} \times \frac{[\text{substrate}]}{[\text{inhibitor}]} = K, \quad \text{where} \quad n = \frac{\text{reaction rate with inhibitor}}{\text{reaction rate without inhibitor}},$$

it was found that succinate oxidation is competitively inhibited by malonate. K , the ratio [succinate]/[malonate] giving 50% inhibition (after correction for the blank respiration), was about 12 (Table 6). It is of interest that this value approximates to the value of about 10 for malonate inhibition of succinoxidase in mammalian muscle tissue, and is completely different from the value of about 60 found by Krebs *et al.* (1952) for yeast.

Table 6. *Malonate inhibition of succinate oxidation*

Each flask contained 3.89 mg. cell N. Substrate concn. as indicated. Other conditions as in Table 5. For explanation, see text.

Succinate M $\times 10^3$	Malonate M $\times 10^4$	Succinate concn. Malonate concn.	$q_{O_2}(N)$ (30–90 min.)		n	K
			Uncorrected	Minus blank		
0.0	0.0	—	2.8	—	—	—
6.25	0.0	—	21.9	19.0	—	—
6.25	50.0	12.5	12.3	9.5	0.50	12.7
6.25	25.0	25.0	15.4	12.6	0.66	12.8
6.25	12.5	50.0	18.3	15.5	0.82	11.4
6.25	6.25	100.0	19.8	17.0	0.89	12.2
0.0	50.0	—	3.6	0.8	—	—

Average value for $K = 12.2$.

Fumarase activity

10 ml. of an aqueous suspension of treated cells was introduced into each of two gas-washing bottles containing 0.5 ml. M-sodium bicarbonate and 4.5 ml. water. The bottles were incubated at 25° and nitrogen containing 5% (v/v) carbon dioxide was continuously bubbled through the suspensions. After 15 min. 15 ml. 0.1 M-sodium fumarate was added to one bottle and 15 ml. water to the other. Samples (5 ml.) were removed from each bottle at 0, 1, 2.5, and 3.5 hr. after the addition of substrate for estimation of malic acid by polarimetric analysis. Each sample was rapidly mixed with 1.25 ml. glacial acetic acid, 6 ml. water, 2.5 ml. M-sodium citrate and 11.25 ml. 29% (w/v) ammonium molybdate. After filtration, readings were taken in a 2 dm. tube using sodium light and the amount of L-malic acid formed was calculated using $(\alpha)_D = +1340^\circ$ for the specific rotation of the malate-molybdate complex in the presence of citrate (Krebs & Eggleston, 1943). The amount of malic acid formed is shown in Table 7. Chromatography of the product confirmed the presence of malic acid.

Decarboxylation and oxidation of α -ketoglutarate

Without the addition of cofactors α -ketoglutarate was not oxidized, though it was decarboxylated. In a system containing treated cells (4.32 mg. cell N), 0.067 M-phosphate buffer, pH 6.5, and 25 μ mole α -ketoglutarate in a total

Table 7. *Fumarase activity*

Gas washing bottles contained: 10 ml. treated cell suspension (25.82 mg. cell N), 0.5 ml. M-NaHCO₃, 4.5 ml. H₂O, and either 15 ml. 0.1 M-Na fumarate or 15 ml. H₂O. Bottles gassed with 95 % (v/v) N₂ + 5 % (v/v) CO₂. Samples (5 ml.) withdrawn at intervals for estimation of malate.

Time of incubation (hr.)	L-Malic acid formed (μ mole)		Conversion of fumarate to malate (%)
	Control	With fumarate	
0	0.0	33.6	2.24
1	9.0	252.9	16.86
2.5	31.3	534.9	35.66
3.5	4.5	702.8	46.85

volume of 2.5 ml., together with 0.2 ml. of 15 % (w/v) potassium hydroxide in the centre wells where necessary, the oxygen uptakes in 4 hr. without and with substrate were 54.3 and 54.5 μ l. respectively. The corresponding carbon dioxide evolutions were 61.8 and 171.1 μ l.

This pattern of gas exchange suggested decarboxylation of α -ketoglutarate at either the α - or γ -positions to produce succinic semialdehyde (β -aldehydopropionic acid) or α -ketobutyric acid, respectively. Chromatograms of the keto-acid 2:4-dinitrophenylhydrazones prepared from the reaction mixture showed two large yellow areas at R_f 0.47 and 0.36. These were hydrogenated and the resulting amino acids run on two-dimensional chromatograms with phenol-ammonia and collidine-lutidine as solvents. The keto-acid hydrazone spot of R_f 0.36 produced no amino acid and its identity remains obscure. The other substance (R_f = 0.47) was reduced to an amino acid identified as γ -aminobutyric acid. The keto-acid from which this arose was thus succinic semialdehyde; α -ketobutyric acid would be reduced to the amino acid α -aminobutyric acid.

Table 8. *Oxidation of α -ketoglutarate*

Warburg flasks contained in a total volume of 2.5 ml.: 4.99 mg. cell N, 0.1 M-phosphate buffer, pH 6.5, 'liver coenzyme concentrate' 2 mg., ATP 2 mg., methylene blue 0.1 mg., 0.01 M-MgSO₄, and, where added, 0.02 M- α -ketoglutarate, 0.005 M-malonate. The centre wells contained 0.2 ml. 15 % (w/v) KOH. Gas phase air; temp. 25°.

	Endogenous	α -Keto-glutarate	Malonate	α -Keto-glutarate + malonate
O ₂ uptake (μ l.) in 240 min.	200.9	272.3	206.8	241.7
O ₂ uptake minus endogenous	—	71.4	5.9	40.8
Malonate inhibition (%):				
(i) endogenous not subtracted	—	—	—	11.2
(ii) endogenous subtracted	—	—	—	42.9

In the presence of methylene blue, 'liver coenzyme concentrate', adenosine triphosphate (ATP) and magnesium sulphate, liquid nitrogen-treated cells oxidize α -ketoglutarate slowly. The rate of oxidation was inhibited by malonate (Table 8). In the presence of malonate some succinate was detected chromatographically. In the absence of malonate no succinate accumulated. (The sample of α -ketoglutarate used contained some succinate as impurity, but this

was insufficient to account for the succinate formed during the oxidation of α -ketoglutarate in the presence of malonate.) The demonstration earlier of a malonate-sensitive succinoxidase system in *Zygorrhynchus moelleri* suggests that inhibition of α -ketoglutarate oxidation by malonate is, in effect, an inhibition of the further oxidation of the succinate formed. It is, nevertheless, worth noting that Price (1953) has found that α -ketoglutarate oxidase from pea stems is inhibited by 0.03 M-malonate.

Oxidation of citrate and cis-aconitate

Both citric and *cis*-aconitic acids stimulated the uptake of oxygen by the cold-treated cells without the addition of co-factors. The rate of citrate oxidation, however, was slow. In an experiment in which each Warburg flask contained: cell material (7.22 mg. cell N), 0.1 M-phosphate buffer, pH 6.5, and 100 μ mole substrate in a total volume of 2.5 ml., with KOH in the centre well, the oxygen uptake in 3 hr. with no substrate, with citrate and with *cis*-aconitate was 78, 121, and 297 μ l., respectively.

Attempts to demonstrate aconitase activity polarimetrically by the formation of *L-isocitric* acid from citric acid, in a manner similar to that of Krebs *et al.* (1952), were not successful. It was possible, however, to show the formation of citrate and α -ketoglutarate from *cis*-aconitate. Chromatography of the organic acids produced in the respiration experiment described above showed the presence of citric acid and α -ketoglutaric acid in the flasks containing *cis*-aconitic acid, but no reaction product from the flasks containing citric acid.

By using the more sensitive technique of keto-acid hydrazone chromatography, the qualitative formation of α -ketoglutarate from both citrate and *cis*-aconitate could be shown in the same system. The amount formed was not determined accurately, but the sizes of the spots of α -ketoglutarate 2:4-dinitrophenylhydrazone produced from equal volumes of the reaction mixture showed a greater formation from *cis*-aconitate than from citrate.

The identity of the α -ketoglutarate was confirmed by hydrogenation of the eluted hydrazones and identification of the resulting amino acid as glutamic acid by chromatography.

Oxidation of L-malic acid

The oxidation of *L*-malic acid was examined at pH 6.5 and 9.0, and also at pH 9.0 in the presence of glycine. Eisenberg (1953), investigating *L*-malate oxidation by dried cells of *Rhodospirillum rubrum*, found that the oxidation had a pH optimum of 9.0 and was inhibited by the oxalacetate formed, or by added oxalacetate. The use of glycine + phosphate buffers at pH 9.0 stimulated the rate of oxidation, due apparently to the decarboxylation of the oxalacetate by the amino group of glycine. Ostern (1933) found that oxalacetate is decarboxylated by the amino group of aniline.

The *Zygorrhynchus moelleri* preparation showed a greater rate of oxidation of *L*-malate at pH 9.0 than at pH 6.5, but the addition of glycine at pH 9.0 caused no significant stimulation (Table 9).

Table 9. *Oxidation of L-malic acid*

Warburg flasks contained in a total volume of 2.5 ml.: 8.27 mg. cell N, 0.08 M-phosphate buffer of the pH indicated below, 'liver coenzyme concentrate' 2 mg., ATP 2 mg., methylene blue 0.1 mg., 0.01 M-MgSO₄, and, where indicated, 0.08 M-glycine (pH 9.0) and 0.04 M-L-malate. The centre wells contained 0.2 ml. 15% (w/v) KOH. Gas phase air; temp. 25°.

Conditions	O ₂ uptake (μ l.) in 180 min.		
	Endogenous (a)	L-Malate (b)	(b) - (a)
pH 6.5	41.6	125.6	84.0
pH 9.0	61.2	185.9	124.7
pH 9.0 + glycine	60.6	190.7	130.1

The products of malic acid oxidation were investigated by chromatography of the keto-acid hydrazones. The experimental conditions were those in Table 9 at pH 6.5 without glycine. An examination was also made of the products accumulating in the presence of 0.02 M-potassium cyanide as a trap for keto-acids. In the presence of cyanide keto-acid 2:4-dinitrophenylhydrazones corresponding to those of authentic samples of pyruvic and oxalacetic acids were obtained, the latter in small quantities only. In the absence of cyanide pyruvate but no oxalacetate was detected. The identity of the pyruvate derivative was confirmed by hydrogenation and comparison of the product with an authentic sample of alanine by chromatography in two solvents. Quantities of the oxalacetate derivative sufficient for positive confirmation of its identity were not obtained: chromatography of the hydrogenated derivative in phenol-ammonia showed a very faint spot corresponding to aspartic acid. Owing to the small amount of material available it was not chromatographed in butanol-acetic acid.

Pyruvate and acetate metabolism

Pyruvate was decarboxylated very rapidly, both aerobically and anaerobically, with the production of acetaldehyde. The latter was identified qualitatively by the production of a violet colour following the addition to the reaction mixture of pyridine followed by sodium nitroprusside. The rate of oxygen uptake with pyruvate was low and, being very much smaller than the rate of carbon dioxide evolution, was difficult to measure accurately. The metabolism of pyruvate and malate together produced a gas exchange less than the sum of the gas exchanges with each substrate separately (Table 10).

Table 10. *Pyruvate, acetate, and L-malate oxidation*

Warburg flasks contained in a total volume of 2.5 ml.: 5.78 mg. cell N, 0.05 M-phosphate buffer, pH 6.5, 0.04 M-substrate, and the supplements as in Table 9. Gas phase air; temp. 25°. Endogenous respiration has been deducted.

	Endogenous	Substrate				
		Malate	Pyruvate	Acetate	Malate + pyruvate	Malate + acetate
O ₂ uptake (μ l.)	167	365	67	6	290	351
CO ₂ output (μ l.)	471	1854	1898	16	3109	1774
R.Q.	2.8	5.1	28.6	2.7	10.7	5.1

Acetate alone appeared not to be metabolized and malate oxidation was not affected by the addition of acetate (Table 10). The addition of cofactors (adenosine triphosphate, 'liver coenzyme concentrate', magnesium, or methylene blue) did not stimulate acetate oxidation.

Formation of citric acid

Liquid nitrogen-treated cells were incubated with malate, pyruvate, acetate, malate plus pyruvate, and malate plus acetate. After 3 hr. samples were taken for citric acid determinations by the method of Natelson, Pincus & Lugovoy (1948). Table 11 shows that most citric acid was formed from a mixture of malate and pyruvate, though the amounts formed from each of these substrates separately were greater than that formed from them both together. The presence of citric acid was confirmed chromatographically, using indicator and the Furth-Hermann reagent as developing sprays.

No citrate was formed from acetate.

Table 11. *Formation of citric acid*

Each boiling tube contained in a total volume of 5.0 ml.: 7.56 mg. cell N, 0.05 M-phosphate buffer (pH 6.5), 'liver coenzyme concentrate' 4 mg., ATP 4 mg., methylene blue 0.2 mg., 0.01 M-MgSO₄, and 0.04 M-substrate. Gas phase air; temp. 25°.

Substrate	Citric acid formed (μ mole)
None (endogenous)	1.05
L-Malate	3.75
Pyruvate	3.10
Acetate	0.85
L-Malate + pyruvate	5.25
L-Malate + acetate	2.40

DISCUSSION

In common with much of the work done on the respiration of organic acids by micro-organisms, the picture presented by whole and cold-treated cells of *Zygorrhynchus moelleri* shows various differences. Owing perhaps to permeability barriers, intact cells of this organism are unable to oxidize the intermediates of the citric acid cycle at pH values near neutrality, whilst exhibiting great oxidative capacities for glucose and acetate. By reducing the pH to 3.4, all the cycle intermediates tested can be oxidized. Inhibitor studies with malonate have shown that at pH 6.8 diethylmalonate in fairly high concentration inhibits the oxidation of both glucose and acetate, though at pH 3.4 succinic acid oxidation is only slightly inhibited by malonic acid (in spite of the latter probably being able to enter the cells), and the inhibition becomes weaker as the experiment proceeds. Gray (1952*a, b*) has observed the adaptive formation of a malonic decarboxylase in *Pseudomonas aeruginosa* which relieved inhibition of succinate oxidation by malonate in that organism.

With cells frozen in liquid nitrogen it has been possible to demonstrate chemically many of the reactions associated with the tricarboxylic acid cycle. These are: the conversion of *cis*-aconitate to citrate, citrate to α -ketoglutarate,

cis-aconitate to α -ketoglutarate, α -ketoglutarate to succinic semialdehyde, α -ketoglutarate to succinate (in the presence of malonate), succinate to a mixture of fumarate and malate, fumarate to malate, malate to oxalacetate (in the presence of cyanide) plus pyruvate, malate to citrate, pyruvate to acetaldehyde, and pyruvate to citrate. Whole cells are able to oxidize acetate very rapidly, but this property is lost when they are frozen, and no reaction products were found from acetate metabolism.

Malonate has been shown to inhibit the succinoxidase system competitively in cells treated with liquid nitrogen. The oxidation of α -ketoglutarate is also inhibited by malonate, but it was not established whether this was due to the inhibition of the α -ketoglutaric oxidase or to inhibition of oxidation of the succinate formed.

Sanadi & Littlefield (1952) and Kaufman, Gilvarg, Cori & Ochoa (1953) found that α -ketoglutarate oxidation requires coenzyme A and diphosphopyridinenucleotide and proceeds via succinyl-coenzyme A; in the absence of an oxidant α -ketoglutarate is decarboxylated slowly to succinic semialdehyde, the latter not being oxidized further by α -ketoglutaric oxidase (Sanadi, Littlefield & Bock, 1952). In view of these findings it is interesting to note that in the absence of coenzyme additions α -ketoglutarate is decarboxylated by *Zygorrhynchus moelleri* with the formation of succinic semialdehyde, but is not oxidized; when cofactors are added oxygen is absorbed and some succinate is formed (Table 8). A similar state of affairs might hold for pyruvate oxidation and acetaldehyde formation (Korkes, del Campillo, Gunsalus & Ochoa, 1951; Korkes, del Campillo & Ochoa, 1952; Littlefield & Sanadi, 1952).

Table 12. *Comparison of oxidative capacities of intact and liquid nitrogen-treated cells*

Comparison of $q_{O_2}(N)$ of intact cells (from Table 1) and $q_{O_2}(N)$ of liquid nitrogen-treated cells (from various tables). Endogenous respiration deducted in each case.

Substrate	Intact cells	Liquid nitrogen-treated cells	Intact cell activity remaining in treated cells (%)
<i>cis</i> -Aconitate	38.0	10.1	26.6
Citrate	197.2	2.0	1.0
L-Malate	295.6	15.8	5.3
α -Ketoglutarate	447.2	3.6	0.8
Pyruvate	530.6	2.9	0.5
Succinate	655.6	23.9	3.6

The oxidative abilities of cells treated with liquid nitrogen are very much less than those of whole cells (Table 12). The high rates of oxidation of some substrates by whole cells, particularly of succinate and acetate (which are oxidized more rapidly than glucose), tend to indicate that these substances are likely to play some part in the oxidative metabolism of glucose by this organism. Krebs (1943), in a similar connexion, remarks: 'Striated muscle is a tissue in which carbohydrate is the predominant substrate for oxidations. . . .

It follows... that a substance which is *rapidly* metabolized in muscle may be expected to be associated with carbohydrate metabolism'.

With frozen cells the position is much less clear. The fraction of the oxidative capacity of the whole cells which remains after treatment with liquid nitrogen hardly exceeds 5%, except in the case of *cis*-aconitate (Table 12), and even here the relatively high percentage of the metabolic activity remaining in treated cells is probably due in part to the low activity shown by whole cells, which in turn may be associated with permeability barriers operative even at low pH values. 'Sparking reactions', such as those found with lupine mitochondria (Brummond & Burris, 1953) and *Azotobacter agile* extracts (Repaske & Wilson, 1953), in which the oxidations of pyruvate and acetate are catalysed by the presence of malate, are not shown either by whole cells or by cells treated with liquid nitrogen (Table 10).

Thus, although work with frozen cells has demonstrated that tricarboxylic acid cycle reactions can be carried out by this organism, any conclusions regarding their quantitative importance must remain tentative.

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REFERENCES

- AJL, S. J. (1951). Terminal respiratory mechanisms in microorganisms. *Bact. Rev.* **15**, 211.
- BEEVERS, H., GOLDSCHMIDT, E. P. & KOFFLER, H. (1952). Use of esters of biologically active weak acids in overcoming permeability difficulties. *Arch. Biochem. Biophys.* **39**, 286.
- BRUMMOND, D. O. & BURRIS, R. H. (1953). Transfer of ^{14}C by lupine mitochondria through reactions of the tricarboxylic acid cycle. *Proc. nat. Acad. Sci., Wash.*, **39**, 754.
- BRYANT, F. & OVERELL, B. T. (1953). Quantitative chromatographic analysis of organic acids in plant tissue extracts. *Biochim. biophys. acta*, **10**, 471.
- CANTINO, E. C. & HYATT, M. T. (1953). Further evidence for the rôle of the tricarboxylic acid cycle in morphogenesis of *Blastocladiella emersonii*. *J. Bact.* **66**, 712.
- CARSON, S. F., FOSTER, J. W., JEFFERSON, W. E., PHARES, E. F. & ANTHONY, D. S. (1951). Oxidative formation of lactic acid by a fungus. *Arch. Biochem.* **33**, 448.
- CASIDA, JUN., L. E. & KNIGHT, S. G. (1954). The oxidation of tricarboxylic acid cycle compounds by *Penicillium chrysogenum*. *J. Bact.* **67**, 658.
- CHUGHTAI, M. I. D., PEARCE, A. A. & WALKER, T. K. (1950). The mechanism of the formation of organic acids by mould fungi. 4. The formation of acetic and pyruvic acids in *Aspergillus niger* growing in glucose media. *Biochem. J.* **47**, 135.
- COCHRANE, V. W. & PECK, JUN., H. D. (1953). The metabolism of species of *Streptomyces*. VI. Tricarboxylic acid cycle reactions in *Streptomyces coelicolor*. *J. Bact.* **65**, 37.
- DANFORTH, W. (1953). Oxidative metabolism of *Euglena*. *Arch. Biochem. Biophys.* **46**, 164.
- DAVIES, D. D. (1953). The Krebs cycle enzyme system of pea seedlings. *J. exp. Bot.* **4**, 173.
- DEFFNER, M. (1942). Über der Stoffwechsel des Schimmelpilzes *Aspergillus niger*. I. Abbau der Citronensäure. *Liebigs Ann.* **552**, 191.

- DENT, C. E. (1948). A study of the behaviour of some sixty amino acids and other ninhydrin-reacting substances on phenol-'collidine' filter-paper chromatograms, with notes as to the occurrence of some of them in biological fluids. *Biochem. J.* **43**, 169.
- EISENBERG, M. A. (1953). The tricarboxylic acid cycle in *Rhodospirillum rubrum*. *J. biol. Chem.* **203**, 815.
- EL HAWARY, M. F. S. & THOMPSON, R. H. S. (1953). Separation and estimation of blood keto acids by paper chromatography. *Biochem. J.* **53**, 340.
- FINCHAM, J. R. S. (1953). Ornithine transaminase in *Neurospora* and its relation to the biosynthesis of proline. *Biochem. J.* **53**, 313.
- FOULKES, E. C. (1951). The occurrence of the tricarboxylic acid cycle in yeast. *Biochem. J.* **48**, 378.
- FRENCH, C. S. & MILNER, H. W. (1951). The photochemical reduction process in photosynthesis. *Symp. Soc. exp. Biol.* **5**, 232.
- GARNER, H. R. & KOFFLER, H. (1951). Preliminary evidence against the existence of a Krebs cycle in *Streptomyces griseus*. *Bact. Proc.* 139.
- GRAY, C. T. (1952*a*). Principles involved in the malonate inhibition of succinate oxidation by bacteria. *Bact. Proc.* 149.
- GRAY, C. T. (1952*b*). The malonic decarboxylase of *Pseudomonas aeruginosa*. *J. Bact.* **63**, 813.
- HALLIWELL, G. & WALKER, T. K. (1952). The metabolism of glucose and acetate in *Aspergillus niger*. *J. exp. Bot.* **3**, 155.
- HOCKENHULL, D. J. D., HERBERT, M., WALKER, A. D., WILKIN, G. D. & WINDER, F. G. (1954). Organic acid metabolism of *Penicillium chrysogenum*. 1. Lactate and acetate. *Biochem. J.* **56**, 73.
- HOLLAND, J. & HUMPHREY, G. F. (1953). Metabolism of *Paramecium caudatum*. I. Respiration. *Aust. J. exp. Biol. med. Sci.* **31**, 291.
- HUGHES, D. E. (1951). A press for disrupting bacteria and other microorganisms. *Brit. J. exp. Path.* **32**, 97.
- KAUFMAN, S., GILVARG, C., CORI, O. & OCHOA, S. (1953). Enzymatic oxidation of α -ketoglutarate and coupled phosphorylation. *J. biol. Chem.* **203**, 869.
- KORKES, S., DEL CAMPILLO, A., GUNSALUS, I. C. & OCHOA, S. (1951). Enzyme synthesis of citric acid. IV. Pyruvate as acetyl donor. *J. biol. Chem.* **193**, 721.
- KORKES, S., DEL CAMPILLO, A. & OCHOA, S. (1952). Pyruvate oxidation system of heart muscle. *J. biol. Chem.* **195**, 541.
- KREBS, H. A. (1943). The intermediary stages in the biological oxidation of carbohydrate. *Advanc. Enzymol.* **3**, 191.
- KREBS, H. A. & EGGLESTON, L. V. (1943). The effect of citrate on the rotation of the molybdate complexes of malate, citramalate, and isocitrate. *Biochem. J.* **37**, 334.
- KREBS, H. A., GURIN, S. & EGGLESTON, L. V. (1952). The pathway of oxidation of acetate in baker's yeast. *Biochem. J.* **51**, 614.
- LITTLEFIELD, J. W. & SANADI, D. R. (1952). Rôle of coenzyme A and diphosphopyridine nucleotide in the oxidation of pyruvate. *J. biol. Chem.* **199**, 65.
- LYNEN, F. & NECIULLAH, N. (1939). Zum Abbau von Bernsteinsäure, Äpfelsäure und Citronensäure durch Hefe. *Liebigs Ann.* **541**, 203.
- MICKELSON, M. N. & SCHULER, M. N. (1953). Oxidation of acetate by *Ashbya gossypii*. *J. Bact.* **65**, 297.
- MILNER, H. W., LAWRENCE, N. S. & FRENCH, C. S. (1950). Colloidal dispersion of chloroplast material. *Science*, **111**, 633.
- MOSES, V. (1954). The effect of ammonia on the oxidation of glucose by *Zygorrhynchus moelleri*. *Biochem. J.* **57**, 547.
- MOSES, V. (1955*a*). Glucose respiration in *Zygorrhynchus moelleri*; the entry of glucose into the cells. *J. exp. Bot.* **6**, 222.

- MOSES, V. (1955*b*). The disappearance of intermediates involved in glucose oxidation during the starvation of the fungus *Zygorrhynchus moelleri*. *Ann. Bot., Lond., N.S.*, **19**, 211.
- NATELSON, S., PINCUS, J. B. & LUGOVY, J. K. (1948). Microestimation of citric acid; a new colorimetric reaction for pentabromacetone. *J. biol. Chem.* **175**, 745.
- NOSSAL, P. M. (1954). Oxidative reactions in cell-free yeast extracts. *Biochim. biophys. acta*, **14**, 154.
- OSTERN, P. (1933). Methode zur Bestimmung von Oxalessigsäure. *Hoppe-Seyl. Z.* **218**, 160.
- POTTER, V. R. (1948). Homogenate technique. *Meth. med. Res.* **1**, 317.
- PRICE, C. A. (1953). Malonate inhibition of α -ketoglutaric oxidase. *Arch. Biochem. Biophys.* **47**, 314.
- REPASKE, R. & WILSON, P. W. (1953). Oxidation of the intermediates of the tricarboxylic acid cycle by extracts of *Azotobacter agile*. *Proc. nat. Acad. Sci. Wash.*, **39**, 225.
- SANADI, D. R. & LITTLEFIELD, J. W. (1952). Rôle of coenzyme A and DPN in the oxidation of α -ketoglutaric acid. *Science*, **116**, 327.
- SANADI, D. R., LITTLEFIELD, J. W. & BOCK, R. M. (1952). Studies on α -ketoglutaric oxidase. II. Purification and properties. *J. biol. Chem.* **197**, 851.
- SHU, P., FUNK, A. & NEISH, A. C. (1954). Mechanism of citric acid formation from glucose by *Aspergillus niger*. *Canad. J. Biochem. Physiol.* **32**, 68.
- DE STEVENS, G., DEBAUN, R. M. & NORD, F. F. (1951). On the mechanism of enzyme action. XLV. The rôle of certain dicarboxylic acids in the formation of oxalic acid by wood-destroying moulds. *Arch. Biochem.* **33**, 304.
- TOWERS, G. H. N., THOMPSON, J. F. & STEWARD, F. C. (1954). The detection of the keto acids of plants. A procedure based on their conversion to amino acids. *J. Amer. chem. Soc.* **76**, 2392.

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