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THE BREAKDOWN OF CARBOHYDRATE BY SCHIZOPHYLLUM COMMUNE FR.

THE OPERATION OF THE TCA CYCLE

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The tricarboxylic acid cycle (TCA cycle), as proposed by Krebs and coworkers, has received widespread consideration in explaining the enzymatic reactions that bring about the breakdown of pyruvate to carbon dioxide and water. As a result of experiments with isolated mitochondria, the concept has arisen that the enzymes of the TCA cycle are localized mainly in mitochondria (cf. SCHNEIDER and HOGEBOOM, 1956). Although the scheme is apparently characteristic of metabolism in animal tissues, there is good evidence that it is at least potentially functional in higher plants, bacteria and yeasts. The operation of the cycle in molds however is still the subject of much investigation and controversy. Various workers have shown the presence of some or all of the individual enzymes of the TCA cycle (cf. COCHRANE, 1958), but relatively little work has been done on the isolation of functionally active subcellular particles from fungi. (BONNER and MACHLIS, 1957). In this report some observations on subcellular particles from *Schizophyllum commune* are given.

METHODS

ORGANISM

A non sporulating mutant strain of *Schizophyllum commune* Fr. (C.B.S. Baarn) was grown for 10 days at 27° on a medium consisting of 10 g glucose; 2 g asparagine; 0.5 g KH_2PO_4; 0.5 g $Na_2HPO_4 \cdot 2H_2O$; 1 g $MgSO_4 \cdot 7H_2O$; 1 ml trace-elements solution (Hoagland and Snijder) per 1000 ml water.

In order to promote growth 10 ml of a yeast-extract preparation was also added and pH adjusted to 7.0. Aeration was obtained by shaking in shallow culture. The submerged mycelium, which was exclusively dicaryontic, was harvested and washed with distilled water.

DISINTEGRATION AND PREPARATION OF CELL-FREE EXTRACTS

30 g prechilled mycelium (wet weight) was ground in a cold mortar with coarse acid-washed sand and a solution containing 0.8 M glucose; 0.05 M KH_2PO_4; 0.02 M nicotinamide (adjusted to pH7.0 with NaOH) during 10 min in the cold. The homogenate was filtered through cloth and centrifuged for 10 min at 800 g. The supernatant

fraction was centrifuged again for 20 min at 24000 g. The resulting supernatant was poured off, and the particulate fraction washed with 30 ml of the disintegration medium, spun down, and then resuspended in 7 ml of this medium. A Phywe refrigerated high-speed centrifuge was used.

Measurements of respiration

Oxygen uptake was measured in standard Warburg experiments at 30°. $QO_2 = \mu LO_2$ taken up/hr/mg dry weight, $QO_2(N) = \mu LO_2$ taken up/hr/mg protein. All values represent averages of duplicate experiments.

Estimation of protein

Protein was estimated spectrophotometrically by the method of Pardee. The protein of the particulate suspension was precipitated by adding one part 10 % trichloroacetic acid, centrifuged, and washed with distilled water. To the washed sediment the biuret-reagent was added and after incubation (45 min at 37°) the mixture was centrifuged, and the optical density measured at 530 mμ in a Beckman spectrophotometer.

Partition chromatography

The reaction mixture of each vessel was deproteinized by adding one drop of 0.5 N HCl and heating in boiling water during 2 min. The protein was spun down and 0.025 ml of the supernatant was spotted on Whatman No 1 paper. Ascending chromatography with n-butanol, acetic acid, water (4:1:5) as solvent during 24 h at 28° has been employed. Spray-reagent slightly alkaline bromophenol blue solution (Lugg and Overell, 1947). The R_f-values of the organic acid spots were compared with those of controls without incubation.

Substrates and coenzymes

Sodium pyruvate and the other TCA cycle intermediates, in the form of acids, were purchased from Fluka, Buchs, Switzerland. The acids were neutralized with an aequivalent amount of K_2CO_3, and shaken with air to remove CO_2. Also from Fluka: adenosine-5-triphosphoric acid or the tetrasodium salt (ATP), coenzyme A (CoA), nicotinamide, and hexokinase (28000 units/g at 30°). Diphosphopyridine nucleotide (DPN), "purest", C. F. Boehringer & Söhne, Mannheim Germany, and cytochrome c (cyt c) Fluka or Boehringer.

RESULTS

Intact mycelium metabolized glucose (0.05 M glucose in 0.05 M phosphate buffer pH 7.0) aerobically as well as anaerobically. The rates were QO_2: —33.4 and $Q^{N_2}_{CO_2}$: +32.5 respectively ($Q^{N_2}_{CO_2} = \mu L\ CO_2$ evolved in nitrogen atmosphere/hr/mg dry weight). Qualitative analyses of the incubation media showed formation of oxalic, citric and lactic acid under aerobic and citric and lactic acid under

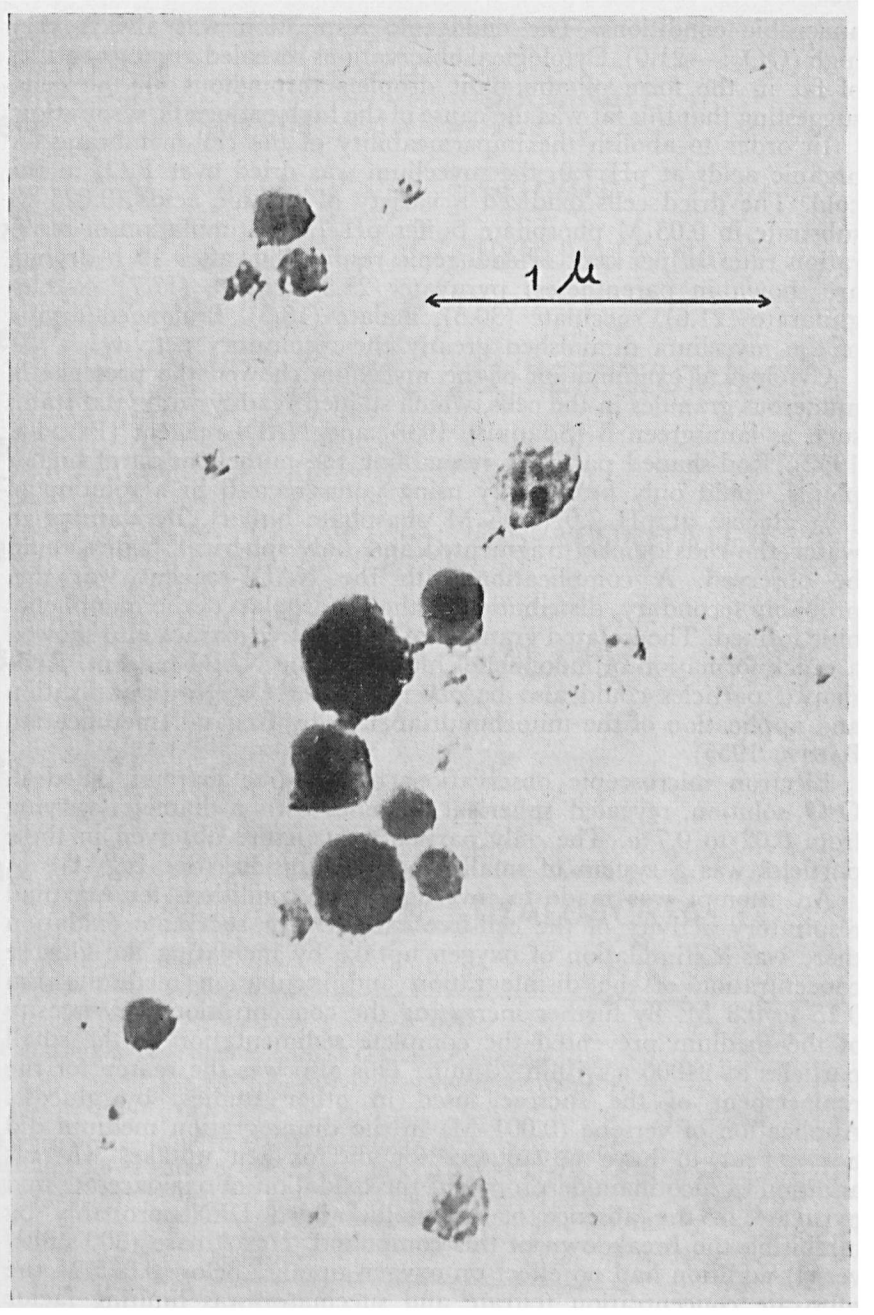

Fig. 1. Electron micrograph: normally prepared suspension of granules, fixed with a solution of OsO_4 in veronal-acetate buffer pH 7.2, (final concentration OsO_4 0.5 %) during 30 min, and washed twice with distilled water.

anaerobic conditions. The endogenic respiration was always very high (QO_2: —21.0). Cytological observations revealed great quantities of fat in the form of abundant droplets throughout all the cells, suggesting that this fat was the cause of the high endogenic respiration.

In order to abolish the impermeability of the cell-membrane for organic acids at pH 7.0, the mycelium was dried over P_2O_5 in the cold. The dried cells oxidized a variety of organic acids (0.025 M substrate in 0.05 M phosphate buffer pH 7.0). Stimulation of respiration rates in per cent of endogenic respiration, after 19 h drying, are shown in parentheses: pyruvate (25.8), citrate (17.7), α-ketoglutarate (21.6), succinate (30.5), malate (18.5). Prolonged drying of the mycelium diminished greatly the respiratory activity.

Cytological examination of the mycelium showed the presence of numerous granules in the cells, which stained readily with vital stains such as janusgreen B (SOROKIN, 1938) and NADI-reagent (PERNER, 1952). Rod-shaped particles, resembling the mitochondria of higher plants, could only be seen by using janusgreen B in a solution of 1 % glucose at pH 7.0 (0.05 M phosphate buffer). By staining in water the rods quickly fragmented and only spherical bodies could be observed. A complication with the NADI-reagent was the, probably secondary, distribution in the fat droplets of the indophenol blue formed. The isolated granules of the cell-free extract also showed a quick formation of indophenol blue with the NADI-reagent. Rod-shaped particles could also be observed after OsO_4-vapour fixation and application of the mitochondrial stain by Regaud (modification BAUTZ, 1955).

Electron microscopic observations on cell-free extracts, fixed in OsO_4-solution, revealed spherical granules with a diameter varying from 0.02 to 0.7 μ. The only particular structure observed in these particles was a system of smaller particles inside. (See Fig. 1).

An attempt was made to investigate the conditions for maximal respiratory activity of the cell-free extract. For succinate oxidation there was a stimulation of oxygen uptake by increasing the glucose concentration of the disintegration and incubation medium from 0.15 to 0.8 M. By further increasing the concentration the viscosity of the medium prevented the complete sedimentation of the small particles at 24000 g within 20 min. This also was the reason for the replacement of the sucrose, used in other studies, by glucose. Application of versene (0.001 M) in the disintegration medium did not appear to have advantages for the oxygen uptake, whereas addition of nicotinamide promoted the oxidation of oxaloacetate and pyruvate in the absence of externally added DPN, probably by inhibiting the breakdown of this compound. Hexokinase (300 units/vessel) addition had no effect on oxygen uptake. Below 0.025 M the substrate concentration (citrate and succinate) was limiting factor in the respiration rate. In all these experiments the not-varied conditions were according to standard experiments as given in Table 1.

In such a typical experiment (Table 1 and Fig. 2) the cell-free extract showed oxidation of all TCA cycle intermediates (isocitrate

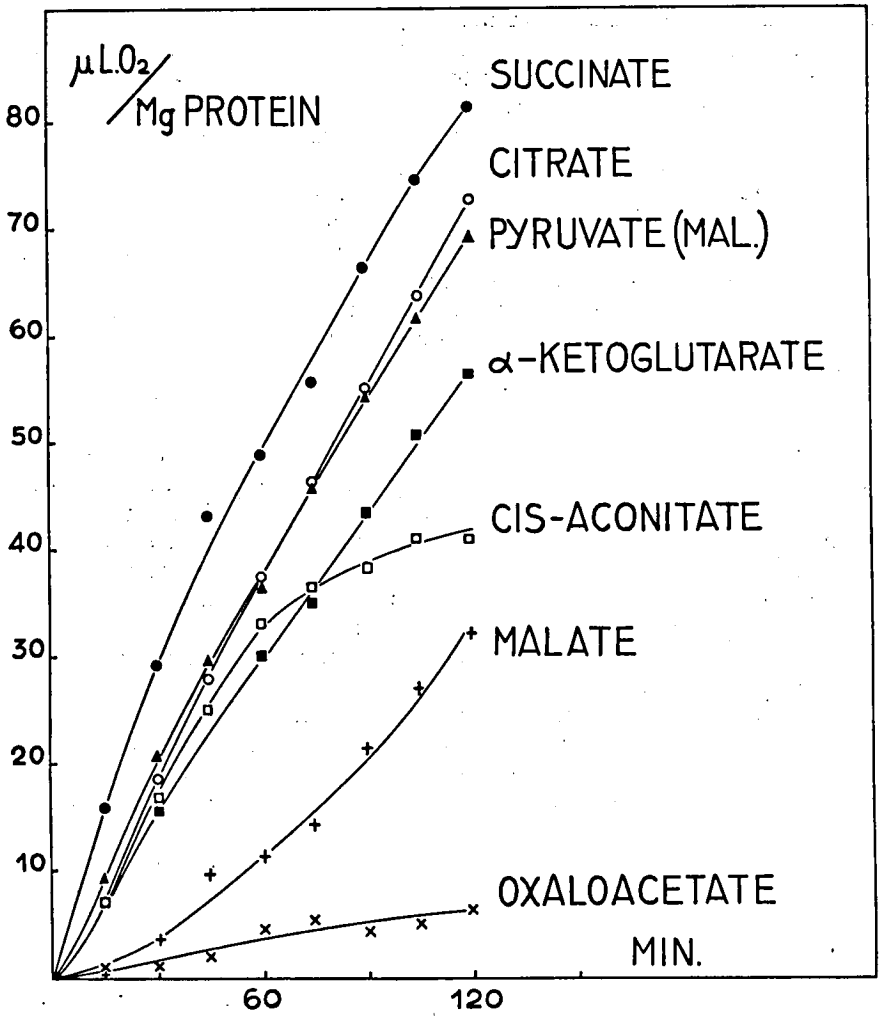

Fig. 2. Oxygen uptake with TCA cycle intermediates by the particulate fraction. Reaction mixture: see table I.

TABLE I

Oxygen uptake with TCA cycle intermediates by the particulate fraction. Reaction mixture: 0.8 M glucose; 0.05 M KH_2PO_4; 0.005 M $MgCL_2$; 0.01 M nicotinamide; 16 × 10^{-6} M cyt c; 0.003 M ATP; 3 mg DPN/vessel; 0.025 M substrate; 0.5 ml cell-free extract; final volume 3.0 ml; pH 7.0.

substrate	QO_2 (N)
pyruvate + 0.001 M malate	37.5
citrate	37.5
cis-aconitate	32.7
α-ketoglutarate	29.7
succinate	49.3
malate	11.3
oxaloacetate	4.0

and fumarate were also oxidized but no exact data are available). Although the enzyme system brought about the oxidation of pyruvate alone (QO_2 (N): —16.9), the addition of a small amount of malate caused a greatly increased rate of oxygen uptake (0.001 M malate alone QO_2 (N): —2.3), suggesting an oxidative condensation of a 2-c and 4-c compound.

Oxaloacetate was oxidized at a very slow rate. One assumption may be put forward: It is possible that externally added oxaloacetate, being a competitive inhibitor of succinic dehydrogenase (PARDEE and POTTER, 1948), inhibits its own oxidation via the reactions of the TCA cycle. This would be a situation analogous to the inhibition of succinic dehydrogenase by malonate, which inhibited not only the oxidation of succinate but also the oxidation of other TCA cycle intermediates (see Table III).

An interesting fact was the oxidation of malate. The oxygen consumption was low, and the curve showed a considerable lag phase, in contrast to the curves for the other acids. An explanation of these results may be put forward on the assumption that the rate of oxygen uptake was limited by the action of lactic dehydrogenase, with or without the "malic enzyme" of Ochoa (SALLES and OCHOA, 1950, KORKES *et al.*, 1950). In the case of lactic dehydrogenase activity this would imply formation of oxaloacetate, subsequent decarboxylation of this compound to pyruvate and a competition of lactic dehydrogenase and oxygen for DPNH. When the "malic enzyme" is also active, there can be a direct formation of lactate from malate via pyruvate, the "malic enzyme" competing with malic dehydrogenase for malate. In these cases one would expect the formation of lactic acid especially in the beginning, whereby the lag in the curve for oxygen uptake is explained.

Furthermore the requirement of the added cofactors was investigated (Table II).

TABLE II

Cofactor requirement of some oxidations by the particulate fraction. Control reaction mixture: as mentioned in table I. Rates of oxygen uptake were calculated on a basis of control = 100.

substrate	control	—DPN	—ATP	—cyt c	+ Co A (2 mg/vessel)
pyruvate + 0.001 M malate	100	45	116	—	208
citrate	100	37	124	—	—
cis-aconitate	100	46	90	—	—
α-ketoglutarate	100	83	33	—	203
succinate	100	160	49	43	—
malate	100	41	15	—	—

It must be noted that the data given in Table II cannot be compared mutually for the different acids, because they are the results of separate experiments. Omission of DPN greatly diminished the oxygen uptake

with all substrates, except succinate. This fact can be explained by the DPN-linked formation of oxaloacetate, and the subsequent inhibition of succinic dehydrogenase by this compound. The omission of cyt.c caused a great decrease of oxygen uptake with succinate as a substrate. Addition of Co A resulted in a 100 % increase of oxygen consumption with pyruvate and α-ketoglutarate.

Some experiments were carried out to study the effect of malonate and arsenite on oxygen consumption with various substrates. Arsenite is known to block the sulphydryl groups such as in lipoic acid, a cofactor in the oxidation of pyruvate and α-ketoglutarate (PETERS *et al.*, 1946).

TABLE III

Influence of malonate and arsenite on oxygen uptake by the particulate fraction. Control reaction mixture: as mentioned in table I. To this medium malonate or arsenite was added in a final concentration of 0.001 M. No oxygen uptake with malonate (0.001 M) alone.

substrate	per cent inhibition	
	+ malonate	+ arsenite
pyruvate + 0.001 M malate	14.9	96.5
citrate	20.3	20.3
α-ketoglutarate	26.7	98.9
succinate	20.0	8.7
malate	5.5	46.0

The results suggest that oxidation of the various substrates is mediated by the reactions of a fully operating TCA cycle. An explanation remains to be found for the relatively low inhibition, by malonate, of the succinate oxidation as compared with citrate and α-ketoglutarate oxidation. It should be noted however that the oxidation of succinate is inhibited already for 20–40 % in the control medium (probably depending on the internal concentration of DPN) by the presence of externally added DPN.

The substrates and products of TCA cycle reactions were separated by filterpaper chromatography. The results are summarized in Table IV. Reaction mixtures after incubation under normal conditions were used (see Table I), inhibited by malonate and arsenite (see Table III), and reaction mixtures in which the glucose concentration was lowered to 0.05 M. In the last case oxygen uptake was diminished as compared with 0.8 M glucose mixtures as shown in parentheses: pyruvate + 0.001 M malate (30.9 %), citrate (37.3 %), α-ketoglutarate (46.9 %), succinate (36.6 %), malate (29.3 %).

It is evident that more reaction products could be isolated by lowering the osmolarity of the reaction mixture, possibly due to a loss of structural integration of the granules. After inhibition with arsenite no reaction products could be detected with pyruvate and α-ketoglutarate as substrates. Noteworthy is also the accumulation of citrate with all the substrates by inhibiting with malonate or by lowering the glucose concentration.

TABLE IV

Results of chromatographic analysis of organic acids formed by the particulate material. Reaction mixtures: as mentioned in table I (0.8 M glucose) respectively with lowered glucose concentration (0.05 M glucose), and addition of malonate or arsenite (see table III). Incubation time 120 min. The added substrate was always present yet on the chromatographs. At zero-time only a substrate spot could be detected.

substrate	0.8 M glucose	0.05 M glucose	0.8 M glucose + malonate	0.8 M glucose + arsenite
pyruvate 0.001 M malate	citrate	citrate	citrate	—
citrate	α-ketoglutarate malate ?	—	—	α-ketoglutarate
α-ketoglutarate	citrate	citrate	citrate	—
succinate	fumarate	citrate fumarate	citrate ? α-ketoglutarate	fumarate
malate	fumarate	citrate succinate fumarate	citrate fumarate	fumarate

DISCUSSION

The present work adds to the evidence for a certain similarity in the intracellular distribution of enzymes in higher fungi and other organisms studied. Dried mycelium and a cell-free extract, consisting of a particulate microscopically visible fraction, was shown to contain the enzymes necessary for the aerobic oxidation of the TCA cycle intermediates. The effect of cofactors and inhibitors, and the products isolated from the reaction mixtures strongly suggest that the TCA cycle is operating indeed in cell-free extracts of *Schizophyllum commune*. The physiological role of this cycle in the intact organism, however, is not yet fully established. The difficulty, how to relate the accumulation of organic acids in the environment by the intact organism under aerobic conditions to the presence of the enzyme system mentioned above, remains to be elucidated. Although the oxygen consumption observed with malate was low in comparison to that with other acids tested (except oxaloacetate), malate readily served as a sparker for pyruvate oxidation suggesting that malate was being oxidized to oxaloacetate. The experimental evidence suggests, however, that malate can be alternatively transfered to lactate. Preliminary observations on oxygen uptake indicated that this alternative mechanism is located mainly in the smaller particles. The results of BROWN and CANTINO (1955) are of interest here. They found that homogenates of *Blastocladiella* will dismutate malate to lactate without appreciable oxygen consumption. In this context it is important that under aerobic conditions *Schizophyllum* produced more lactic acid from glucose

than anaerobically, a phenomenon which was pointed out for other fungi by CARSON *et al.*, (1951), BROWN and CANTINO (1955) and GENTILE (1954). A sequence in which reactions of the TCA cycle and an enzyme system such as the "malic enzyme" of Ochoa are involved will offer a plausible explanation for this aerobic formation of lactic acid.

This proposed mechanism for the formation of lactic acid, via the reactions of the TCA cycle needs, however, further investigation, and consideration must be given to the operation of a glyoxylic acid cycle (cf. KORNBERG and KREBS, 1957) or another mechanism for the production "de novo" of 4-carbon compounds.

SUMMARY

Some observations have been made on a strain of *Schizophyllum commune Fr.*, concerning the activity of normal and dried mycelium and a cell-free extract containing a microscopically visible fraction. The results fit in with the assumption of a TCA cycle, operating in this fungus. The possible role of this cycle in the accumulations of lactic acid has been discussed.

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